DETECTION OF METHICILLIN RESISTANT STRAINS OF STAPHYLOCOCCUS AUREUS USING PHENOTYPIC AND GENOTYPIC METHODS IN A TERTIARY CARE HOSPITAL

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CERTIFICATE

This is to certify that this dissertation titled **"DETECTION OF METHICILLIN RESISTANT STRAINS OF STAPHYLOCOCCUS AUREUS USING PHENOTYPIC AND GENOTYPIC METHODS IN A TERTIARY CARE HOSPITAL"** is a bonafide record of work done by **Dr.N. LAKSHMI PRIYA,** during the period of her Post graduate study from June 2006 to March 2009 under guidance and supervision in the Institute of Microbiology, Madras Medical College and Government General Hospital, Chennai-600003 in partial fulfillment of the requirement for **M.D.Microbiology** Degree Examination of The Tamilnadu Dr. M.G.R. Medical University to be held in March 2009.

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DECLARATION

I declare that the dissertation entitled ***"DETECTION OF METHICILLIN RESISTANT STRAINS OF STAPHYLOCOCCUS AUREUS USING PHENOTYPIC AND GENOTYPIC METHODS IN A TERTIARY CARE HOSPITAL"*** *submitted by me for the degree of M.D. is the record work carried out by me during the period of* ***January 2007 to January 2008*** *under the guidance of* ***Dr.G. SUMATHI M.D., Ph.D.,*** *Director and Professor, Institute of Microbiology, Madras Medical College, Chennai. This dissertation is submitted to The Tamilnadu Dr.M.G.R. Medical University, Chennai, in partial fulfillment of the University regulations for the award of degree of M.D., Branch IV (Microbiology) examination to be held in March 2009.*

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CONTENTS

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Introduction

INTRODUCTION

Skin and soft tissue infection (SSTIs) are common type of infection that may contribute to longer hospital stays, significantly increase the cost of medical care, and are likely to have an important role in the development of antimicrobial resistance.[65] Most of these infections are superficial and readily treated with a regimen of local care and antibiotics. However, soft tissue infections involving deeper layers like fascia and muscle can rapidly progress to systemic sepsis and prove fatal.[95] *Staphylococcus aureus* is one of the common organisms associated with soft tissue infection.[88]

S. aureus are ubiquitous organisms and among the most commonly encountered in medical practice. *S. aureus* has been reported as a major cause of community and hospital acquired

infections.[93] The organisms has a differential ability to spread and cause outbreaks in hospitals.[113] Infections caused by *S. aureus* used to respond to β-lactam group of antibiotics. Penicillin – resistant *S. aureus* strains began emerging shortly after introduction of penicillin in 1940.Resistance to methicillin and other β-lactamase – resistant Penicillin was first observed in *S.aureus* soon after methicillin was introduced into clinical use in 1961.[92]

methicillin resistant *Staphylococcus aureus* (MRSA) are significant pathogen that have emerged over the past 30 years to cause both nosocomial and community acquired infections. There has been a steady increase in the prevalence of MRSA isolated in hospitals. Indian literature shows that MRSA incidence was as low as 20% in 2002 (Supriya et al 2002) and reached to 43% in 2008 (Erics et al 2008)

The prolonged hospital stay, indiscriminate use of antibiotics and the lack of awareness are possible predisposing factors for MRSA emergence.[5] Serious endemic and epidemic MRSA infections occur globally as infected and colonized patients in hospitals mediate the dissemination of these isolates and hospital staff assists further transmission.[1]

Many of the MRSA strains are multidrug resistant and are susceptible only to glycopeptides antibiotics such as vancomycin.[84] Therefore the knowledge of prevalence of MRSA and their current antimicrobial profile becomes necessary in the selection of appropriate empirical treatment of these infections.

Expression of methicillin resistance in clinical laboratories is subject to environmental conditions like pH, incubation time, temperature and salt concentration of the medium[85]. Conditional expression of PBP2a may cause ambiguity in susceptibility tests. To complicate matters further, methicillin resistance is often expressed heterogeneously in that only 10^8 cells of the population are phenotypically resistant. These factors emphasize the need to develop a rapid, standard, accurate and sensitive method for detection of methicillin resistance in *Staphylococcus aureus* which is not dependent on growth conditions[39].

The conventional methods to detect MRSA in the laboratory include oxacillin disc diffusion, cefoxitin disc diffusion, MIC determination by agar or broth dilution method and oxacillin screen agar method. Numerous approaches that improve the turn around time for the detection of MRSA have been described. PCR is considered the gold standard; it is rapid with a high degree of sensitivity and specificity[12].

The sooner an MRSA infection is diagnosed and the susceptibility to antimicrobial agents established, the earlier appropriate therapy and control measures can be initiated. Identification and antimicrobial susceptibility testing are therefore crucial steps in treating, controlling and preventing MRSA infection.

Hence this study has been undertaken to compare the conventional methods of detecting MRSA infection with the molecular technology and also to determine the antimicrobial susceptibility pattern of MRSA in a tertiary care hospital which will facilitate in the implementation of appropriate treatment of the patients.

Review of Literature

REVIEW OF LITERATURE

HISTORICAL PERSPECTIVE

In 1884, Rosenbach provided the first formal description the genus *Staphylococcus* and divided the genus into two species *Staphylococcus aureus* and *Staphylococcus albus*. In 1885, Passet added a third species *Staphylococcus citreus*.[92]

In 1989, the genus *Staphylococcus* was brought under the broad *Bacillus – Lactobacillus – Streptococcus* cluster consisting of Gram – positive bacteria that have a low G + C Content of DNA.[14]

The closest relatives of *Staphylococci* are the *Macrococci* and on the basis of partial oligonucleotide sequencing, *Staphylococci* are also related to *Salinococci, Enterococci, Planococci, Bacillus* and *Listeria*.[92]

DEFINITION OF GENUS

Members of the genus *Staphylococcus* are Gram positive cocci measuring 0.5 – 1.5 µm, that occurs singly, in pairs in tetrads, short chains and irregular grape like clusters. They are non – motile, non spore forming, catalase positive, facultatively anaerobic and are often unencapsulated. [31]

Staphylococcus are susceptible to furazolidone (100µg) and are resistant to low levels of bacitracin (0.04 units). They are susceptible to lysis by lysostaphin and are relatively resistant to lysis by lysozyme.[92]

Staphylococcus is currently composed of 37 species, 16 of which are found in humans. The most virulent ones include *S. aureus* and *S. lugudensis* in humans and *S. aureus* and *S. intermedius* in animals.[103]

HABITAT

S. aureus are ubiquitous colonizers of the skin and mucosa of virtually all animals, including mammals and birds.[73] *S. aureus* demonstrates a preference for the anterior nares especially in adults.[80] It can exist as a resident or as a transient member of the normal flora. Nasal carrier rate may vary from 10% - 40% in both the community and the hospital environment.[110] Nasal carriage of *S. aureus* has also become a means of persistence and spread of multiresistant *Staphylococcus* especially MRSA.[108]

CELL MORPHOLOGY AND CULTURAL CHARACTERISTICS : LIGHT MICROSCOPY

Gram stained cells of *Staphylococcus* are uniformly Gram positive in young cultures and appear spherical with an average diameter of 0.5 – 1.5µm.[92] Cell wall defective or deficient (L – form) cells have been described for *S. aureus*. [54]

CULTURAL CHARACTERISTICS

Colonies of most *Staphylococcus* species grow rapidly and are 1-3 mm in diameter after 24 hours of incubation. *Staphylococcal* colonies are usually smooth,butyrous and have a low convex profile with a entire edge. Pigmentation is characteristic of this species when grown aerobically and ranges from cream through buff to gold. Pigmentation is enhanced on fatty media such as Tween agar, by prolonged incubation

and by leaving plates at room temperature .*Staphylococcus aureus* is tolerant of concentrations of sodium chloride that inhibit other bacteria and on Mannitol salt agar it forms 1mm diameter yellow colonies surrounded by yellow medium due to acid formation. On MacConkey or Cysteine Lactose Electrolyte Deficient agar (CLED) it acquires the appropriate colour of the indicator,depending on whether or not the particular strain ferments lactose.[8,31]

Several selective media have been devised for isolating *S. aureus*. These include mannitol salt agar. Salt milk agar, salt broth, lipase – salt – mannitol agar, phenyl ethyl alcohol agar, Columbia Colistin Nalidixic acid (CNA) agar, Baird –Parker agar base.[92]

Severe unusual morphotypes of *S. aureus* have been described that depart significantly from the normal colony morphology and include certain encapsulated strains[112], L – forms[54] and small colony variant (SCV)[92]. SCV's are believed to have a defect in the electron transport and are resistant to aminoglycosides.[115] They are most commonly isolated from persistent infections such as cystic fibrosis or chronic osteomyelitis.[63]

BIOCHEMICAL REACTIONS

Staphylococcus aureus are:

Catalase positive

Hydrolyse urea

Reduce nitrates to nitrites

Liquefy gelatin

Methyl Red and Voges Prosakeur test positive

Indole test – negative

Produce phosphatase

Hydrolyse DNA

Slide coagulase and Tube coagulase test positive

Reduce Tellurite to form black colonies in Potassium tellurite medium

Produce themostable nucleases

They ferment a number of sugars producing acid but no gas. Sugar fermentation is of no diagnostic value except for mannitol, which is usually fermented by *S. aureus* but not by other species.[31,92,8]

PATHOGENESIS OF *S. AUREUS* INFECTIONS

S. aureus expresses many cell surface associated and extra cellular proteins that are potential virulence factors[92].

a) Adherence protein

S. aureus expresses on their surface the following protein that promote attachment to host proteins[77].

1) Fibronectin binding protein A & B

2) Clumping factor A & B

3) Collagen binding protein

4) Bone sialoprotien binding protein

5) Elastin binding protein

6) Plasmin sensitive protein

7) Biofilm associated protein

b) Avoidance of host defense :

i) Capsular polysaccharide – serotype 5 or 8

It is also called as Microcapsule because it can be visualized only by Electron microscopy after antibody labelling.

2) Protein A

It is a surface protein of *S.aureus* which binds to the Fc region of Ig-G and disrupts opsonization and phagocytosis[102]

c) Damage to hosts[92] ;

I Membrane damaging toxins :

1) α - toxin – cytolytic pore forming toxin

2) β – toxin – neural sphingomyelinase

3) δ - toxin - surfactant on various cells

4) γ - toxin –Bicomponent toxin

Cytolytic for erythrocytes and leukocytes

5) Panton Valentine Leukocidin – cytolytic for leukocytes

II Pyrogenic toxin –Superantigens

1) Enterotoxins – Ingestion of performed toxin in food results in nausea, vomiting and diarrhoea

2) TSST – 1 – Toxic Shock Syndrome Toxin

3) Exfoliative toxin A & B – Staphylococcal scalded skin syndrome

III) Enzymes[92] :

i) Fibrin forming and fibrinolytic enzymes

a) Staphylocoagulase : - It reacts with coagulase reacting factor in the plasma and the resulting complex, Staphylothrombin converts fibrinogen to fibrin.

b) Staphylokinase – Binds to plasminogen and activates it to become the fibrinolytic enzyme plasmin.

ii) Bacteriolytic enzyme

Endo – β – N – acetyl glucosaminidase

Lysotaphin endopeptidase

iii) Hydrolytic enzymes

Lipase

Thermonuclease

Urease

Hyaluronidase

Protease

CLINICAL SYNDROMES

In general, infection begin with the colonization of target tissues by the microbes. *S. aureus* mainly colonizes the anterior nares. Further infection results from more specific invasion processes, during which bacteria interacts directly or indirectly (through toxins) with the host[80].

S. aureus causes a wide range of infections. These can be broadly divided into community and hospital acquired infections[92].

Community acquired infection include the following :

1) Toxin-mediated disease[61] :- Staphylococcal scalded skin syndromes

Toxic shock syndrome

Staphylococcal food poisoning

2) Skin and soft tissue infections[92] :

The basic pathologic lesion induced by *S. aureus* is a pyogenic exudate or an abscess. *S. aureus* infection of skin and soft tissues are classified according to the anatomic structure involved :-

Impetigo

Folliculitis

Furuncles, carbuncles Erysipelas, cellulitis, fascitis

3) Bone and joint sepsis;

S. aureus is the leading cause of primary septic arthritis and osteomyelitis[7].

4) Bacteremia and endocarditis :

Around two – third of patients with *S. aureus* bacteremia are nosocomially acquired most of which are associated with intravenous device related infection. Infective endocarditis on a native valve is one of the most severe complications of *S. aureus* bacteremia[35].

II. Nosocomial or Hospital acquired infection[43];

Surgical wound infection

Ventilator associated pneumonia.

Bacteremia associated with intravenous devices

Infection associated with prosthetic material such as CSF shunts, prosthetic joints and vascular grafts.

GENE REGULATION

S. aureus gene expression is under the control of regulatory systems that respond to change in environmental conditions. The best characteristics of the regulatory systems are the Agr (Accessory global regulator) and Sar (Staphylococcal accessory regulator)[75].

PLASMIDS

Plasmids are facultative extrachromosomal genetic elements which are common in natural population of most *Staphylococcal* species[111]. *S. aureus* plasmids have been classified into three genera classes I,II and III . Class I plasmid usually encodes a single antibiotic resistance[74]. Class II plasmid is commonly referred to as penicillnase or β – lactamase plasmids[94]. Class III plasmid encode resistance to gentamicin, tobramycin and kanamycin[41].

BACTERIOPHAGES

Typing of *S. aureus* strains by an official International basic set of phages found wide use as an established method for epidemiology[114]. Human strains of *S. aureus* belong to phage groups I, II, III or V[107]. Phage typing was the mainstay of typing for many years but has been replaced by Ribotyping, Pulse field gel electrophoresis(PFGE), Multilocus enzyme electrophoresis (MLEE), Plasmid profiling and Multilocus sequence typing (MLST)[92].

RESISTANCE TO ANTIMICROBIAL AGENTS

The rising level of resistance to a wide range of antibiotics by *S. aureus* represent a significant threat to future treatment efficacy[92].Since the beginning of the antibiotic era, *S. aureus* has

responded to the introduction of new drugs by rapidly acquiring resistance by a variety of genetic mechanisms including[64].

1) Acquisition of extrachromosomal plasmids or additional genetic information in the chromosome via transposons or other types of DNA insertion.
2) By mutations in chromosomal genes

RESISTANCE OF STAPHYLOCOCCI TO B-LACTAM ANTIBIOTICS:

Penicillin resistant *S. aureus* strains began emerging shortly after the introduction of penicillin in medicine in the early 1940's. Today the percentage of penicillin – resistant strains has risen to 75-95% with the highest rates being found among the hospital strains[92]. Most penicillin resistant *Staphylococcal* strains produce β-lactamase which hydrolyses the β-lactam ring of the antibiotic rendering the β-lactam inactive. β-lactamase genes (blaz) are most often found on class II plasmids. Blaz gene is under the control of two adjacent regulatory genes, the antirepressor bla R1 and the repressor blaI [55].

METHICILLIN RESISTANCE

a) HISTORY AND EPIDEMIOLOGY

Resistance to Methicillin and other β-lactamase resistant Penicillins was first observed in *S. aureus* soon after methicillin was introduced into clinical use in Britain[52]. The methicillin resistant strains

isolated in Britain at that time came from hospitalized patients, which were multiple antibiotic resistant, belonged to phage group III and their resistance to methicillin was heterogenous affecting only a minority of the cell population[9].

After the mid 1970's large outbreaks of infection by MRSA were recorded in many hospitals[92]. Many of these outbreaks appear to have been caused by a single epidemic strain that was transferred between hospitals by the movement of patients[28]. A nomenclature has been developed to differentiate strains associated with epidemic MRSA from those associated with sporadic infections which do not appear to have the same degree of transmissibility. MRSA is now responsible for around 30% of more of all serious *S. aureus* infections[92].

The Indian scenario

One of the earliest reports from India was 1982, where an incidence of 26.6% was reported by Bhatia et al[10]. In 1996, Pullimood et al., from Vellore reported an MRSA isolation rate of 24%[78]. The following a Mabel from Thiruvananthapuram reported an MRSA isolation rate of 16.4%[9], while Udhaya Shankar et al., reported the figure as 20% from Pondicherry[106].

A systematic study done in Delhi over six months period by Krishna Prakash et al in 2001 reported an MRSA isolation rate of 38.6%[59]. In 2003, Hanumanthappa et al., reported an isolation rate of 43%[46]. A higher rate of 54.85% has been reported in the same year by Anupurba S et al., from Banaras[1].

The therapeutic outcome of infections that result from MRSA is worse than the outcome of those that result from methicillin sensitive strains. The difference has been ascribed to the underlying medical problem as well as the less effective bactericidal drugs available to treat these infections, rather than to enhanced virulence of the MRSA strains[52]. Like the penicillin – resistant strains, the MRSA isolates also frequently carry resistance genes to other antimicrobial agents[36].

MECHANISM OF RESISTANCE

Methicillin resistance requires the presence of the chromosomally localized *mec A* gene[55].The *mec A* gene is responsible for the synthesis of penicillin binding protein 2a (PBP2a : also called PBP2) – a 78 KDa protein[19]. Pencillin binding proteins (PBPs) are membrane bound enzymes that catalyzes the transpeptidation reaction that is necessary for cross – linkage of peptidoglycan chains[55]. PBP2a substitutes for the other PBPs and because of its low affinity for all β-lactam antibiotics, enables *Staphylococci* to survive exposure to high

concentration of these agents. Thus, resistance to methicillin confers resistance to all β-lactam antibiotics including cephalosporins . PBP2a differs from other PBPs in that its active site blocks binding of all β-lactams but allows the transpeptidation reaction to proceed[36].

The *mec A* is part of a genomic island designated Staphylococcal Cassette Chromosome mec (SCC mec)[56]. To date, four different SCC mec elements have been characterized[50]. The recent upsurge of CA-MRSA infections reported in patients from different countries was associated with the detection of a unique SCC mec,type IV. This element, smaller than the other elements, appears more genetically mobile and does not carry additional antimicrobial resistance genes[26].

Phenotypic expression of methicillin resistance is variable[105]. Expression in the MRSA strain is regulated by genes mecI and mecRI. An additional series of genes, the *fem* genes (factors essential for methicillin resistance), play a role in crosslinking peptidoglycan strands and also contribute to the heterogeneous expression of methicillin resistance[2].

Since no homologue of *mec A* exists in methicillin susceptible *Staphylococcus aureus*, it has been assumed that *mec A* was acquired from one of several Coagulase negative Staphylococcal species[50]. A

mec A homolog with 88% amino acid similarity to *mec A* MRSA has been identified in *S. sciuri*[24].

QUINOLONE RESISTANCE

Fluoroquinolones were initially introduced for the treatment of Gram negative bacterial infection in 1980. However, because of their Gram positive bacterial spectrum, they have also been used to treat bacterial infections caused by *Staphylococci.* Quinolone resistance among *S. aureus* emerged quickly, more prominently among the methicillin – resistant strains[36].

Three mechanisms of fluroquinolones resistance have been postulated for *Staphylococci*

1) Mutations in chromosomal gene *gyr A,* encoding the DNA gyrase, so that its function is no longer inhibited by the antibiotic[97].
2) Mutations in chromosomal gene *norA* that encodes a membrane efflux protein for hydrophilic fluoroquinolone[117].
3) Mutations in chromosomal gene *grl A* that encodes the DNA topoisomerase IV[34]

When quinolones are used to treat infections caused by other bacterial pathogens, subjects colonized with *S. aureus* are likely exposed to subtherapeutic antibiotic concentration and therefore at risk

of becoming colonized with resistant mutants[47]. These resident resistant strains then become the reservoir for future infection[49].

RESISTANCE OF *S. AUREUS* TO VANCOMYCIN:

The glycopeptide antibiotics vancomycin and teicoplanin bind to the peptidyl – D alanyl D alanine terminus of peptidoglycan precursors and prevent the transglycosylation steps of cell wall peptidoglycan synthesis. Since this mode of action is different from that of β-lactams, glycopeptides are being used to treat severe infections caused by methicillin and other β-lactam resistant Staphylococci[92].

The dramatic increase in the use of vancomycin to treat infections caused by methicillin resistant Staphylococci and Enterococcal infections preceeded the emergence of vancomycin resistant Staphylococci.[32] Staphylococci resistant to vancomycin in a clinical isolate was first reported in a strain of *S. hemolyticus*[58].

In 1997, the first report of vancomycin intermediate resistant *S. aureus* (VISA) came from Japan[29]. The first vancomycin resistant *S. aureus (*VRSA) was reported in Michigan in July 2002[17]. In contrast to the chromosomally mediated resistance for VISA strains VRSA acquire resistance by conjugal transfer of the *Van A* operon from *Enterococcus faecalis*[47].

RESISTANCE TO MACROLIDE, LINCOSAMIDE AND STREPTOGRAMIN : - (**MLS**)

Methicillin resistant Staphylococci often have cross resistance to Macrolides (erythromycin, spiramycin, clarithiomycin, azithriomycin), lincosamides (lincomycin, clindamycin) and streptogramin type B antibiotics – designated as MLS resistant. The different types of MLS antibiotics bind to the 50S ribosomal subunit and interfere with transpeptidation and translocation reactions needed for peptide chain elongation[92].

RESISTANCE TO TETRACYCLINE

This is widespread among Staphylococcus species and ranks along with β-lactams and MLS resistance as one of the most frequent types of antibiotic resistance[91]. There are two mechanisms for tetracycline resistance found in Staphylococci. The most common one involves an energy dependent pumping of tetracycline from the cell so that levels of these antibiotics are reduced below that required to inhibit the ribosome[23]. The second mechanism involves ribosome protection so that protein synthesis is unaffected by the presence of tetracycline, doxycycline or minocycline[90].

RESISTANCE TO AMINOGLYCOSIDES

There are three major mechanisms responsible for aminoglycoside resistance in *S. aureus* . One mechanism involves changes in ribosomal protein as a consequence of certain mutations in their structural genes such that ribosomes can no longer bind streptomycin[104]. A second mechanism involves energization and permeability of cell membrane. The third and most common mechanism of resistance involves modification of aminoglycosides by aminoglycoside modifying enzymes so that the antibiotics are no longer capable of binding to ribosomes[92].

RESISTANCE TO TRIMETHROPIM

Trimethoprim resistance is due to overproduction of the native dihydrofolate reductase or a reduced affinity of the native DHFR for trimethoprim or a trimethoprim resistant DHFR[92].

LABORATORY DIAGNOSIS

Direct microscopic examination of normally sterile fluids – CSF, joint aspirates and pus aspirated from deep sites, may provide a rapid, presumptive report of Gram positive cocci resembling Staphylococci.

Isolation of Staphylococci from primary clinical specimens is usually performed using blood agar – eg. Tryptic soy agar supplemented with 5% sheep blood, followed by incubating at 35-37°C

for 18-24 hours. Most Staphylococcal species will produce abundant growth and the colonies are usually 1-3 mm in diameter, golden yellow pigmented, hemolytic, circular, smooth and raised with a butyrous consistency[92]. Screening for the presence of *S. aureus* in mixed cultures such as nasal swabs is often performed using mannitol salt agar, Columbia colisitin – nalidixic acid agar, lipase – salt – mannitol agar or phenyl ethyl alcohol agar. These media inhibit the growth of Gram negative organisms but allow Staphylococci to grow[92].

S. aureus is identified by its colony morphology. Gram stain demonstrates the presence of Gram positive cocci in clusters and it also gives a positive catalase test[8,92]. *S. aureus* strains are usually identified by

1) Slide and tube coagulase test
2) Mannitol fermentation
3) Gelatin liquefaction
4) Phosphatase production
5) DNA hydrolysis
6) Thermonuclease test
7) Latex agglutination test detecting the presence of protein A

MOLECULAR METHODS:

Most molecular methods for identification of *S. aureus* have been PCR based[27]. A range of primers designed to amplify species specific targets have now been developed[66]. Such targets include the

Nuclease (nuc)

Coagulase (Coa)

16s ribosomal RNA

Surface protein A (Spa)

Factors essential for methicillin resistance - *fem A & fem B*

DETECTION OF METHICILLIN RESISTANT *S. aureus* :

In assessing the performance of susceptibility testing method, the Minimum Inhibitory Concentration (MIC) determination by the dilution method has traditionally been the reference method. MIC methods have now been replaced as the reference methods by molecular methods which detect the *mec* A gene. Disc diffusion methods remains the most widely used in routine clinical laboratories[27].

I) DISC DIFFUSION METHODS

Standardized methods have been defined by the Clinical Laboratory Standards (CLSI)[25]. Oxacillin disc (1µg) is used to determine methicillin resistance[70]. Cefoxitin disc (30 µg) diffusion tests are more reliable than those with Oxacillin[27].

2) MIC DETERMINATION

1) Agar dilution method

2) Broth -Micro dilution method

-Macro dilution method

3) E - test

Procedure

Serial dilution of Oxacillin is added onto Mueller Hinton agar / broth with 2% Nacl. A young peptone water culture of *S. aureus* corresponding to 0.5 McFarland turbidity is used as the inoculum and it is incubated at 33-35°C for 24 hours[71].

Oxacillin MIC ≤ 2 μg/ml – susceptible

> 2 μg/ml – resistant

E – test method

E – test also known as the epsilometer test is an exponential gradient testing methodology where E in E test refers to the Greek symbol Epsilon. The E test which is a quantitative method for antimicrobial susceptibility testing applies both the dilution of antibiotic and diffusion of antibiotic into the medium. A predefined stable antimicrobial gradient is present on a thin inert carrier strip. When this E test strip is applied onto an inoculated agar plate,,there is an immediate release of the drug. Following incubation,a symmetrical inhibition ellipse is produced. The intersection of the inhibitory zone

edge and the calibrated carrier strip indicates the MIC value over a wide concentration range with inherent precision and accuracy[62].

3) AGAR SCREENING METHOD

This method recommends the use of Mueller Hinton agar with 4% Nacl and 6μg/ml of oxacillin. The test organism corresponding to 0.5 Mc Farland turbidity standard is inoculated as a spot and the plates are incubated at 35°C for 24 hours. Any growth on the agar plate is indicative of resistance[69].

4) LATEX AGGLUTINATION TESTS

A rapid slide latex agglutination test based on the detection of PBP2a is available. This methods involves extraction of PBP2a from suspensions of colonies and detection by agglutination with latex particles coated with monoclonal antibodies to PBP2a. This test is very sensitive and specific with *S. aureus* but may not be reliable for colonies grown on media containing Nacl[13].

5) MOLECULAR METHODS

The earliest molecular methods for the detection of *mec A* gene relied on either radiolabelled or digoxigenin (DIG) labeled DNA probes[4]. More recently PCR – based methods have been used routinely by reference laboratories as their standard methods for detecting the *mec A* gene[12]. The addition of a second set of primers to

amplify a gene which is always present within Staphylococci like *nuc, gyr A and Coa*g gene has been a very common method[118].

DIRECT IDENTIFICATION OF MRSA IN BLOOD CULTURES

This uses molecular methods for direct detection of MRSA in blood cultures in order to facilitate rapid diagnosis of MRSA and enables appropriate therapeutic decision to be made in a timely manner. Such methods have included gel – based and real time PCR, DNA proper and peptide nucleic acid probes[27].

DETECTION OF MRSA IN SCREENING SAMPLES

Enrichment media are used to enhance the detection of MRSA by overnight incubation before plating on solid agar. Most of this media contain an indicator to distinguish *S. aureus*, inhibitory substance to aid the selection of *S. aureus* from other organisms and drugs like methicillin, oxacillin or more recently cefoxitin[79].

MANAGEMENT OF *S. AUREUS* INFECTIONS[36]

The antibiotic used will depend on the susceptibility of the infecting strain.

1) Pencillin susceptible *S. aureus* – Benzyl Penicillin
Penicillin V and
Amoxicillin can be used

2) Methicillin susceptible *S. aureus*

- Pencillinase resistant penicillins such as cloxacillin, dicloxacillin, flucloxacillin, oxacillin,
- methicillin and naficillin
- I generation cephalosporins
- penicillin / beta lactamase inhibitors
    - Amoxycillin / clavulanic acid
    - Ticarcillin / clavulanic acid
    - Ampicillin / Sulbactam

3) Methicillin Resistant *S. aureus*

- Vancomycin is the drug of choice for serious infections caused by *S. aureus* that are methicillin Resistant

Alternatives - Teicoplanin
Clindamycin
Rifampicin,
Fluoroquinolones,
Fusidic acid,
Co – trimoxazole

4) Vancomycin resistant *S. aureus*

Quinupristin – Dalfopristin and Linezolid are the newer antimicrobial agents currently available

Daptomycin, a novel bactericidal agent that damages cytoplasmic membrane is currently undergoing clinical trials.

PREVENTION OF *S. AUREUS* INFECTION:

Falls into three main categories

1) Eradication of *S. aureus* carriage
2) Hospital infection control measures to prevent nosocomial infection, including preoperative antibiotic prophylaxis
3) Vaccination strategies

ERADICATION OF *S. AUREUS* CARRIAGE

There is considerable evidence to indicate that nasal carriage of *S. aureus* and the development of Staphylococcal infection are related. Rates of infection are higher in carrier than in non carriers including the development of post- operative wound infections[92].

Topical antibiotics such as 2% Mupriocin applied to the nose or to exit sites of prosthetic devices is effective. Topical Lysostaphin has been tried in animal models in eradicating *S. aureus* nasal colonization[92].

HOSPITAL INFECTION CONTROL MEASURES

Regular alcohol based hand washing, preoperative antibiotic prophylaxis is also important in preventing surgical sepsis[92].

VACCINATION

The most significant human Antistaphylococcal vaccine published to date is a single dose of conjugate vaccine comprising *S. aureus* type 5 and type 8 capsular polysaccharides conjugated to non toxic recombinant *Pseudomonas aeruginosa* exotoxin A[92]

Aims of the Study

AIMS OF THE STUDY

1) To detect MRSA strains from patients with soft tissue infection

2) To compare the phenotypic and the genotypic identification methods in the detection of MRSA for their sensitivity, specificity and accuracy.
3) To compare the antimicrobial susceptibility pattern of MRSA and MSSA isolates.
4) To detect susceptibility pattern of the isolates to vancomycin by disc diffusion method and E-test.

Materials & Methods

MATERIALS AND METHODS

Study period

This cross sectional study was carried out from January 2007 – January 2008.

Place of study

Institute of Microbiology, Madras Medical College, Chennai – 03

ETHICAL CONSIDERATION

Ethical and research clearance was obtained from the Institute of Ethical Committee Government General Hospital and Madras Medical College,Chennai-3. Permission to conduct the study was sought from the respective hospital authorities. Informed consent was obtained from the patients before enrolment into the study

STATISTICAL ANALYSIS

Statistical analyses were carried out using Statistical Package for Social Sciences (SPSS) and Epi – info softwares.

SAMPLE

Pus samples were collected from skin and soft tissue infections from patients admitted in general surgery and allied specialities at Govt. General Hospital, Chennai.

SAMPLE PROCESSING

The collected pus samples were subjected to direct Gram stain and inoculated onto nutrient agar, blood agar and Mac Conkey agar. The plates are incubated at 37°C and inspected after overnight incubation.

S. aureus was identified by its colony morphology , Gram stain and catalase test.

Further confirmation was done by slide and tube coagulase test, growth on mannitol salt agar and DNase test by standard microbiological techniques as recommended by CLSI guidelines.

1) SLIDE COAGULASE TEST

The colony is emulsified in a drop of saline on a clean glass slide. A drop of sterile human plasma is added to it and mixed with a loop. A positive test is indicated by clumping of the organisms in suspension. If the suspension is smooth, the test was reported as negative. With each test a positive and negative control was put up[8].

2)TUBE COAGULASE TEST

The colony is suspended in 1ml of 1 in 6 diluted sterile human plasma. The tubes are incubated at 37^0c and examined at the end of 1,2and 4 hours. The tube is gently tilted to see the coagulam formation. If no coagulam is formed at the end of 4 hours ,the tube is reincubated at room temperature for 24 hours[8].

3) MANNITOL SALT AGAR

The organism is inoculated into mannitol salt agar and incubated at 37ºC for 24 hours. *S. aureus* produces yellow coloured colonies. A positive control was put up using *S. aureus* ATCC 25923[8].

4) DEOXYRIBONUCLEASE (DNase) TEST

DNase is detected by heavily spot inoculating several colonies of the organism on DNase test medium. After 24 hours of incubation at 37ºC, the plate is flooded with 3.6% hydrochloric acid. After few minutes, the medium under and around the inoculum becomes clear indicating hydrolysis of DNA[8].

DETECTION OF METHICILLIN RESISTANCE

I) **DISC DIFFUSION METHOD**

Testing for Methicillin resistance was done by using 1 µg Oxacillin and 30 µg Cefoxitin by Kirby Bauer disc diffusion method and incubating at 35ºC for 24 hours. The results are interpreted as per CLSI standards[25].

	SUSCEPTIBLE	INTERMEDIATE	RESISTANT
Oxacillin (1 µg)	≥ 13 mm	11-12 mm	≤ 10 mm
Cefoxitin (30 µg)	≥ 22 mm	NA	≤ 21 mm

ii) OXACILLIN SCREEN AGAR

Reactants

Mueller – Hinton agar with 4% Nacl

Oxacillin stock solution

Procedure

Oxacillin working solutions 6 µg/ml is prepared and added to Meuller – Hinton agar with 4% Nacl and poured into petridishes and allowed to set. A young peptone water culture of *S. aureus* corresponding to 0.5 Mc Farland turbidity standard is prepared and spot inoculated on the agar. The plates are incubated for 24 hours at 35°C.

Results and interpretation

The appearance of even a single colony on oxacillin screen agar indicates oxacillin resistance[27].

III) DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION OF OXACILLIN BY BROTH MICRODILUTION METHOD[71]:

Diffusion tests, widely used to determine the susceptibility of organisms isolated from clinical specimens have their limitations. Thus when in doubt, the way to a precise assessment is to determine the Minimum Inhibitory Concentration (MIC) of the antibiotic to the organisms. The MIC of oxacillin for *S. aureus* was done by broth microdilution method.

Principle

The Minimum Inhibitory Concentration (MIC) is the least amount of antimicrobial that will inhibit visible growth of an organism after overnight incubation. The Minimum Bactericidal Concentration (MBC) is the amount of agent that will prevent growth after subculture of the organism to antibiotic free medium.

Procedure

MIC was determined by using Mueller Hinton broth with 2% Nacl as the medium in a microtitre plate. Serial dilutions of Oxacillin were prepared in distilled water. The concentration of Oxacillin used were : 256,128 , 64, 32, 16, 8,4,2,1,0.5,0.25,0.125 ug/ml. A young peptone water culture of *S. aureus* corresponding to concentration of $2x10^5$ /ml is used as the inoculum. A quality control strain of *S. aureus* was also included. The microtitre place was incubated at 35°C for 24 hours.

Results and interpretation

MIC is expressed as the highest dilution which inhibited the growth as judged by the lack of turbidity in the tube. Standard strain of known MIC run with the test is used as the control to check the reagents and conditions.

MIC > 2 µg / ml – Methicillin Resistant *S. aureus*

≤ 2 µg/ ml – Methicillin Susceptible *S. aureus*

IV.DETERMINATION OF *mec A* GENE BY MULTIPLEX PCR METHOD:

The *mecA* gene is highly conserved among *Staphylococcal* species, therefore,presently,detection of this gene by polymerase chain reaction (PCR) is considered as “gold standard” for detection of methicillin resistance in Staphylococci[12]. Molecular diagnostic assays based on the detection of the *mecA* gene encountered difficulty in discriminating MRSA from methicillin resistant *coagulase negative Staphylococcus* species (MR-CoNS)because the *mecA* gene is widely distributed in *S aureus* as well as in MR-CoNS[85].

In this study multiplex PCR was used which allows the detection of MRSA by using primers specific for methicillin resistance and *coag*ulase genes. The *coag* gene was used to differentiate between *S aureus* and *CoNS*,a gene which allows species specific identification of *S aureus* .In addition MRSA harbours the *mecA* gene encoding methicillin resistance, which is absent in methicillin susceptible Staphylococci[85].

Preparation of cell lysate

Cell lysates of the isolates were used as DNA template for colony lysate PCR. Around 5-10 colonies were suspended in 100µl of milli Q water and boiled for 5 minutes .It is then centrifuged at 10,000 rpm for 10 minutes. The supernatant provided templates for PCR reactions[58].

Primers

Two sets of primers were used for multiplex PCR. The first pair of primers was derived from the region of the *mecA.*

Forward primer- (5”AAAATCGATGGTAAAGGTTGGC) 1282 to 1303

Reverse primer - (5”AGTTCTCAGTACCGGATTTGC) 1793 to 1814.

The second pair of primers was derived from the region of the *coag gene.*

Forward primer - (5”CGAGACCAAGATTCAACAAG)

Reverse primer - (5”AAAGAAAACCACTCACATCAGT).

PCR Reaction Mixture

Components	**Final Concentration of reagents**	**Quantity of reagents**
Water	-	13.3 µl
deoxy Nucleoside Tri Phosphate (d NTP)	(5mM)	2µl
PCR buffer	10x reaction buffer(500Mm, KCL, 100 mM Tris – HCL, pH-8.3)	2µl

mecA gene Forward Primer Reverse Primer	-	 0.25µl 0.25µl
Coag gene Forward Primer Reverse Primer	-	 0.25µl 0.25µl
TAQ Polymerase	2U	0.66µl
Template	-	1µl
Total Volume		**20µl**

Forty amplification cycles were performed with an automated thermocycler according to the following format:

Intial denaturation for 5 min at 95^0 c;denaturation for 30s at 94^0 c;annealing for 30s at 55 ^{0}c;and extension for 90s at 72^0 c. The final cycle was followed by an additional 5 min at 72^0 c to complete partial polymerizations. Amplified products were run using horizontal 1.5%agarose gel electrophoresis. The gel was visualized using a UV transilluminator. The amplified PCR products and 100 base pair DNA molecular markers were seen as bright fluorescent bands.

Interpretation

A 533 bp corresponds to *mec A* and 810 bp corresponds to *coag* gene specific oligonucleotides.

DETECTION OF ANTIMICROBIAL SUSCEPTIBILITY PATTERN

The sensitivity to common antibiotics was done by Kirby – Bauer disc diffusion method as recommended by CLSI.

Test Inoculum – 0.5 Mc Farland lawn culture

Control strains – *S. aureus* ATCC – 25923

The antibiotics used (discs were procured from Himedia)

ANTIMICROBIAL AGENT (µG)	INHIBITION ZONE IN MM		
	RESISTANT ≤	INTERMEDIATE	SENSITIVE ≥
1) Penicillin G 10 units	28	-	29
2) Ampicillin 10 µg	28	-	29
3) Cephalexin 30 µg	14	15-17	18
4) Cefaclor 30 µg	14	15-17	18
5) Cefotaxime 30 µg	14	15-22	23
6) Erythromycin 15 µg	13	14-22	23
7) Chloramphenicol 30 µg	12	13-17	18
8) Gentamicin 10 µg	12	13-14	15
9) Amikacin 30 µg	14	15-16	17
10) Ciprofloxacin 5 µg	15	16-20	21
11) Ofloxacin 5 µg	14	15-17	18
12) Vancomycin 30 µg	-	-	15
13) Teicoplanin 30 µg	10	11-13	14
14) Rifampicin 5 µg	16	17-19	20
15) Linezolid 30 µg	-	-	21

Plates were incubated at 37°C for 16 – 18 hours. The diameters of the zones of inhibition for various drugs were interpreted according to CLSI standards.

DETERMINATION OF VANCOMYCIN RESISTANCE BY DISC DIFFUSION METHOD

Testing for vancomycin resistance was done by using 30 ug vancomycin disc by Kirby Bauer disc diffusion method. The zone of inhibition is read after incubation at 37^0c for 24 hours .The results were interpreted as per CLSI standards.

MINIMUM INHIBITORY CONCENTRATION DETERMINATION FOR VANCOMYCIN BY E – TEST

E – test is based on a combination of both diffusion and dilution tests. It is a quantitative technique for determining the antimicrobial sensitivity.

Principle

It consists of a strip made of an inert material with 8 extensions that carry the discs of 4mm, resembling the “tooth of comb”. Towards the stem of the strip, MIC reading scale in µg/ml is given. A defined concentration of antibiotic is loaded on each of the disc so as to form a

gradient when placed on agar plate. The gradient remains stable after diffusion and the zone of inhibition takes the form of ellipse.

Procedure

A 0.5 Mc Farland turbidity standard of *S. aureus* was inoculated as a lawn culture on Mueller Hinton agar with 2% Nacl .E – strips were placed on the agar surface and plates were incubated at 35^{o}C for 24 hours.

Results and interpretation

MIC was read where the ellipse intersects the scale.

MIC of vancomycin	≤ 4 µg/ml – susceptible
	8-16 µg/ml – intermediate
	≥ 32 µg/ml – Resistant strains

Results

RESULTS

This cross sectional study was carried out during the period Jan 2007 to Jan 2008 in the Institute of Microbiology, Madras Medical College, Chennai.

Methicillin resistance was determined for a total of 150 *S. aureus* isolates from pus samples by oxacillin and cefoxitin disc diffusion method, oxacillin screen agar, MIC – broth micro dilution method, and PCR for *mec A* gene.

Vancomycin resistance was determined by disc diffusion and E – test method.

The antimicrobial susceptibility pattern to various groups of antibiotics were also done by Kirby Bauer disc diffusion method, The results are as follows:

TABLE 1
GENDER DISTRIBUTION OF PATIENTS N = 150

SEX	NO. OF PATIENTS	PERCENTAGE

Male	107	71.3
Female	43	28.7

Table 1 shows that there is male predominance among the cases .

TABLE 2

AGE GROUP DISTRIBUTION OF PATIENTS N = 150

Age	No. of patients	Percentage
0 – 20	14	9.3
21 – 40	84	56
41 – 60	42	28
> 60	10	6.6

Table – 2 shows that the maximum number of isolates were in the age group of 21 – 40 years (56%) followed by 41 – 60 years (28%).

TABLE – 3

DISTRIBUTION OF THE SAMPLE SOURCE N = 150

Speciality	No. of cases	Percentage
1. Orthopedics	51	34
2. General Surgery	42	28
3. Neuro Surgery	17	11.3
4. Dermatology	30	20
5.Cardiothoracic Vascular Surgery	4	2.7
6. Otorhino layrngology	4	2.7
7. Gastroenterology	2	1.4

Most of the isolates were from orthopedics patients 51 (34%) followed by general surgery 42 (28%).

TABLE - 4

RESULTS OF METHICILLIN RESISTANCE IN *S.AUREUS* AS DETERMINED BY OXACILLIN (1UG)DISC DIFFUSION METHOD

Pattern of Resistance	No. of cases	Percentage
1) Susceptible	65	43.3
2) Intermediate	5	3.3
3) Resistant	80	53.3

53.3%of the strains were found to be resistant, 3.3% were intermediate and 43.3% were susceptible strains.

TABLE - 5

RESULTS OF METHICILLIN RESISTANCE IN *S,AUREUS* AS DETERMINED BY CEFOXITIN (30µG) DISC DIFFUSION METHOD .N=150

Pattern of Resistance	No. of cases	Percentage
1) Susceptible	69	46
2) Resistant	81	54

46% of strains were sensitive and 54% were resistant by cefoxitin disc method.

TABLE - 6

RESULTS OF METHICILLIN RESISTANCE IN *S.AUREUS* AS DETERMINED BY MIC OF OXACILLIN-MICROBROTH DILUTION METHOD N=150

Pattern of Resistance	No. of cases	Percentage
Susceptible	69	46
Resistance	81	54

46% of strains were susceptible and 54% were resistant by microbroth dilution method

TABLE - 7

DISTRIBUTION OF OXACILLIN MIC VALUES AMONG *S.AUREUS* N=150

MIC µg/ ml	No. of cases	Percentage
> 256	12	8
128	16	10.7
64	16	10.7
32	14	9.3
16	10	6.6
8	9	6
4	5	3
2	2	1.3
1	2	1.3
0.5	8	5.3
0.25	48	32
0.125	8	5.3

TABLE - 8

PATTERN OF OXACILLIN RESISTANCE AMONG MRSA ISOLATES
N = 82

Oxacillin Resistance Pattern	MIC µG/ML	No. of cases	Percentage
High-level	≥ 32 µg/ml	58	71
Moderate level	≤ 16 ≥ 8 µg/ml	19	23
Low level	4 µg/ml	5	6

Among MRSA strains ,high level oxacillin resistance ≥32 µg/ml were found in 58 strains (39%). Moderate level resistance ≤ 16 ≥ 8 µg/ml were found in 19 strains (13%). Low level resistance 4 µg/ml were found in 5 strains (3%).

TABLE - 9

RESULTS OF METHICILLIN RESISTANCE IN *S.AUREUS* AS DETERMINED BY OXACILLIN SCREEN AGAR (6 µG/ML – OXACILLIN).

Growth on Oxacillin Screen Agar	No. of cases	Percentage
Present	81	54
Absent	69	46

54% of strains of S aureus were methicillin resistant and 46% were susceptible as detected by oxacillin screen agar method.

TABLE 10

RESULTS OF *COAG* GENE DETECTION BY PCR N=50

Coag gene	Total no of cases	Percentage
Positive	50	100
Negative	--	_

The *coag* gene fragment was observed in all strains.

TABLE- 11

RESULTS OF *MEC A* GENE DETECTION BY PCR N =50.

mec A	Total no. of cases	Percentage
Positive	29	58
Negative	21	42

MRSA strains were unequivocally detected within three hours using multiplex PCR with *coag* and *mec A* gene specific oligonucleotides. For MRSA strains ,two discrete DNA fragments,a 533 base pair(bp) *mec A* and 810 base pair(bp) *coag* specific products were obtained. .Multiplex PCR detected 29 strains (58%) as methicillin resistant and 21 strains (42%) as methicillin susceptible.

TABLE- 12

COMPARISON OF RESULTS OF PHENOTYPIC AND GENOTYPIC METHODS IN THE DETECTION OF MRSA N = 50

Oxacillin disc diffusion method			Cefoxitin disc diffusion method		Oxacillin screen agar		MIC by Micro broth dilution method		*mec A* gene analysis	
S	I	R	S	R	S	R	S	R	Pos	Neg

24	1	25	24	26	24	26	24	26	29	21

TABLE -13

Cefoxitin disc diffusion / oxacillin screen agar / microbroth dilution	Polymerase Chain Reaction mecA Positive	Negative	Total
Positive	26	0	26
Negative	3	21	24
Total	**29**	**21**	**50**

Sensitivity - 90%

Specificity - 100%

Positive Predictive value - 100%

Negative Predictive value - 88%

Percentage of false Positives - 0%

Percentage of false Negatives - 10%

Kappa - 0.88

Accuracy - 94%

TABLE – 14

ANTI MICROBIAL SUSCEPTIBILITY PATTERN OF MRSA N = 81

Antibiotics	Susceptible (%)	Intermediate (%)	Resistant (%)
Penicillin			100
Cefotaxime		4	96
Cephalexin	6		94
Ampicillin	8		92
Gentamicin	16.4	3.6	80

Erythromycin	10.5	9.5	80
Cefaclor	22		78
Ciprofloxacin	11	12	77
Co – trimoxazole	36		64
Amikacin	30.5	25.6	44
Ofloxacin	49	11	40
Chloramphenicol	78.1	7.3	14.6
Rifampicin	85.4	7.3	7.3
Vancomycin	100		
Teicoplanin	100		
Linezolid	100		

Among MRSA strains all strains (100%) were resistant to penicillin. More than 80% strains were resistant to Cefotaxime, cephalexin, cefaclor ampicillin,gentamicin,erythromycin. Moderate level of resistance was detected in ciprofloxacin,co-trimoxazole,amikacin and ofloxacin. However the strains were highly sensitive to chloramphenicol and rifampicin. 100% sensitivity were observed to vancomycin, teicoplanin and linezold.

TABLE – 15

ANTI MICROBIAL SUSCEPTIBILITY PATTERN OF MSSA n = 69

Antibiotics	Sensitive (%)	Intermediate (%)	Resistant (%)

Penicillin	4.4		95.6
Ampicillin	22		78
Cephalexin	38		62
Cefotaxime	75		25
Cefaclor	71		29
Erythromycin	52.4	17.6	31
Chloramphenicol	92.6		8.8
Co – trimoxazole	46		54
Gentamicin	57.3	4.4	39.7
Amikacin	60.2	22	17.6
Ciprofloxacin	53	17.6	30.8
Ofloxacin	59.7	13.2	28.5
Vancomycin	100		
Teicoplanin	100		
Linezolid	100		
Rifampicin	98		2

95.6%strains were resistant to penicillin. Moderate level of resistance were seen to ampicillin,co-trimoxazole and cephalexin. However majority of strains were sensitive tocefotaxime, erythromycin, chloramphenicol ciprofloxacin, ofloxacin and rifampicin. All strains were sensitive to vancomycin,teicoplanin and linezolid.

TABLE - 16

RESULTS OF VANCOMYCIN RESISTANCE DETERMINED BY DISC DIFFUSION AND E TEST METHOD N = 150.

Pattern of resistance	No. Of cases	Percentage
Susceptible	150	100’
Intermediate	-	-
Resistant	-	-

All strains were sensitive to vancomycin by disc diffusion and E-test method.

FIGURE – 1

GENDER DISTRIBUTION OF PATIENTS N=150

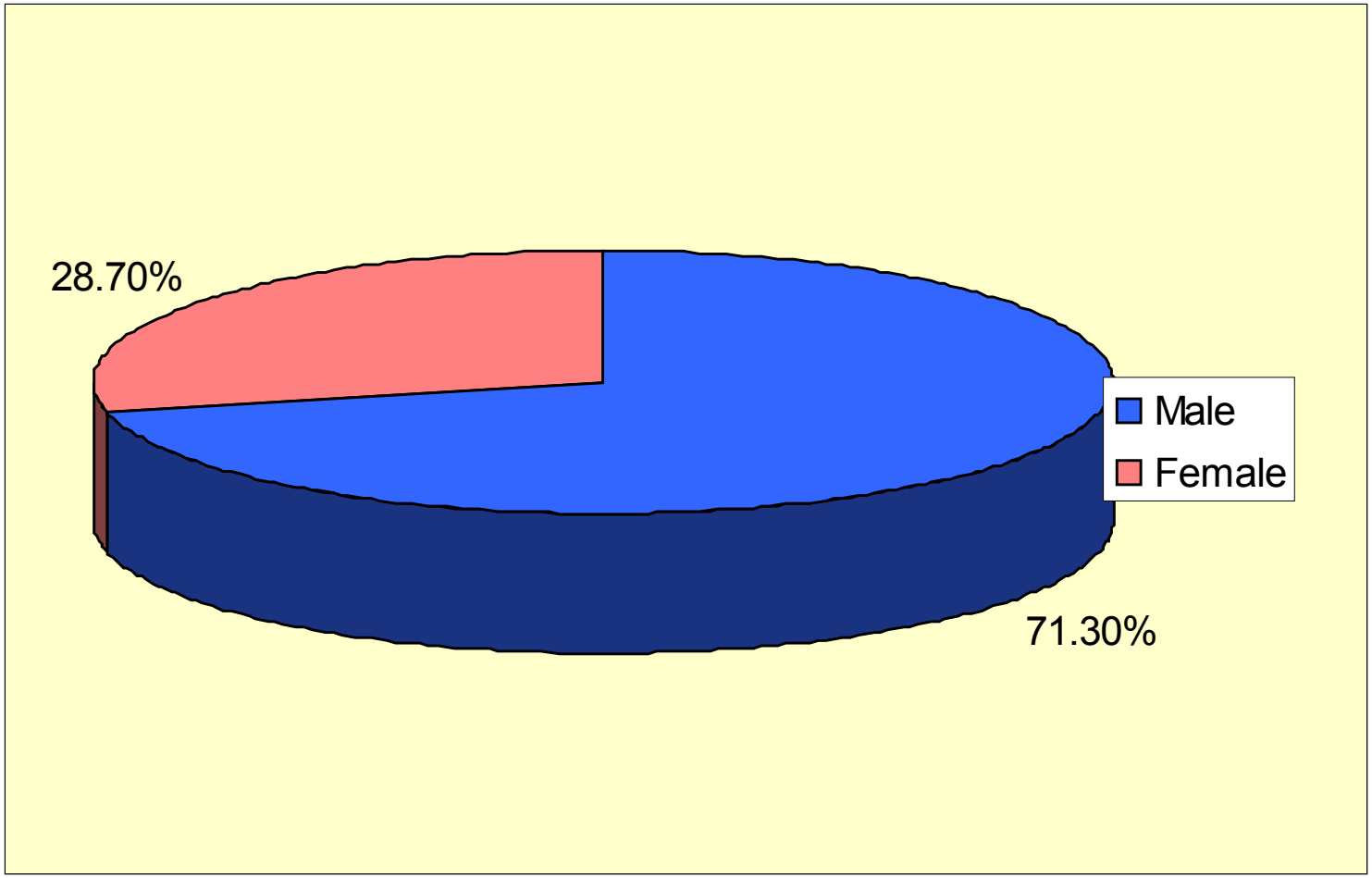


FIGURE – 2

AGE GROUP DISTRIBUTION OF PATIENTS N=150

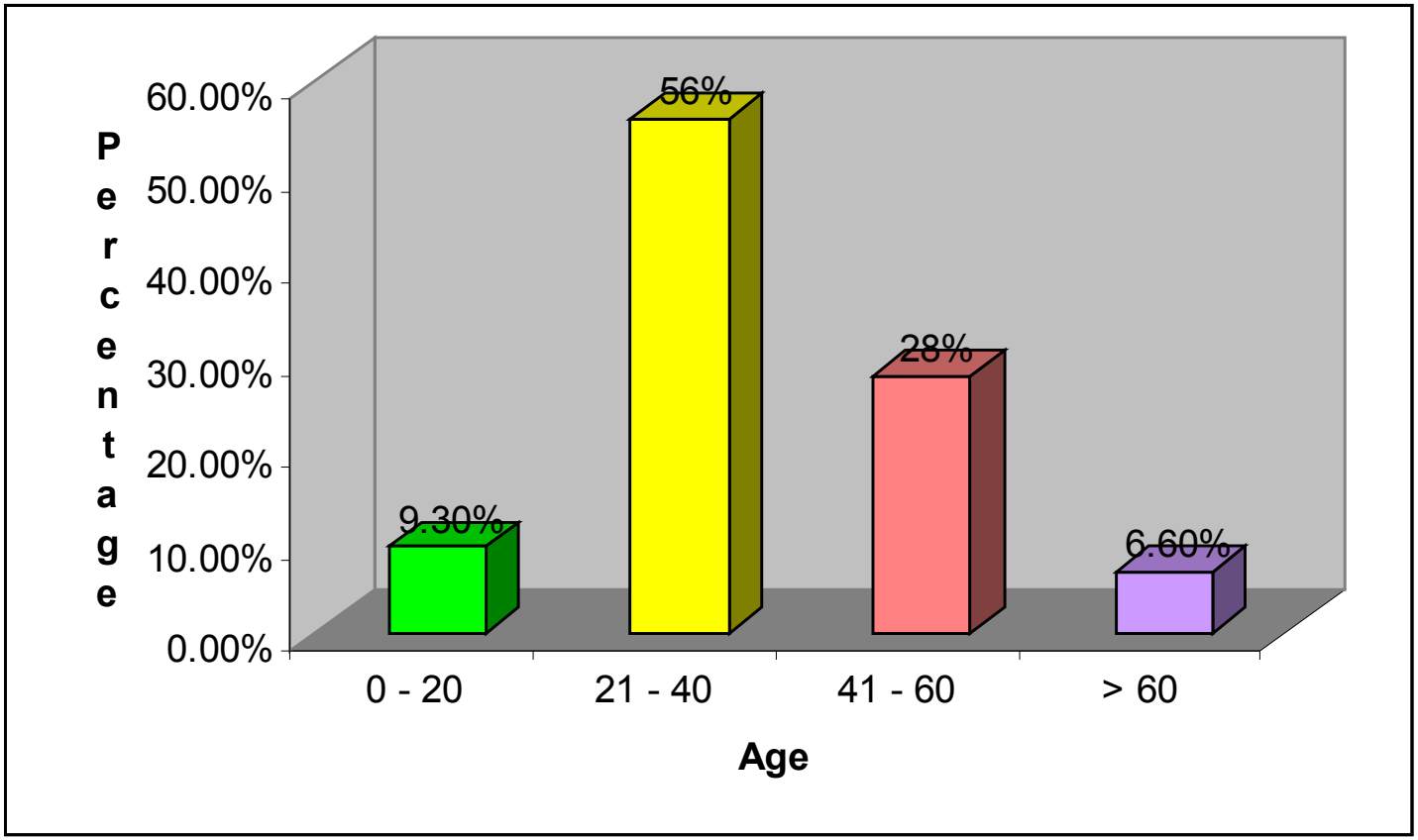


FIGURE – 3

DISTRIBUTION OF THE SAMPLE SOURCE N = 150

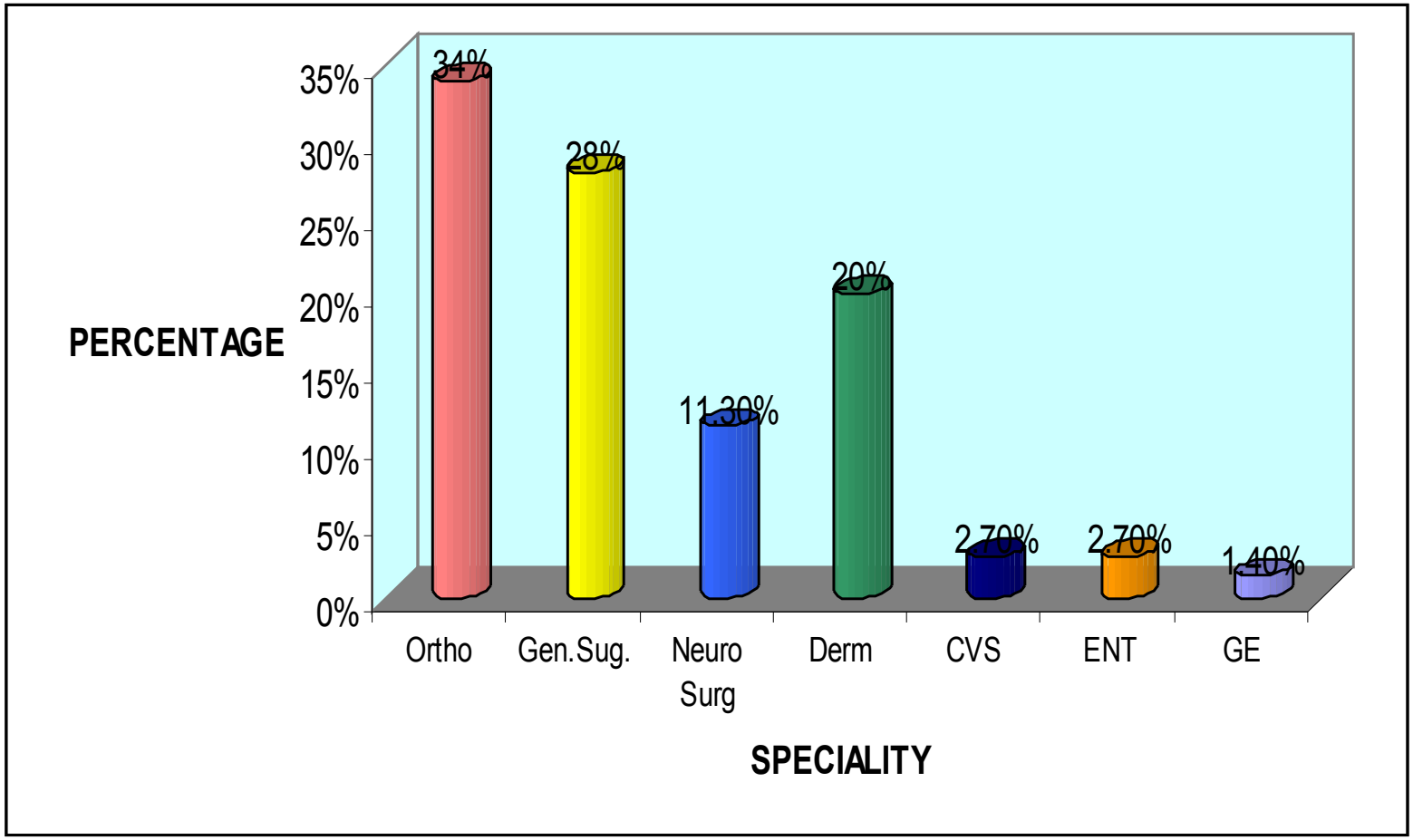


FIGURE – 4

RESULTS OF METHICILLIN RESISTANCE IN S.AUREUS AS DETERMINED BY OXACILLIN (1ug) DISC DIFFUSION METHOD

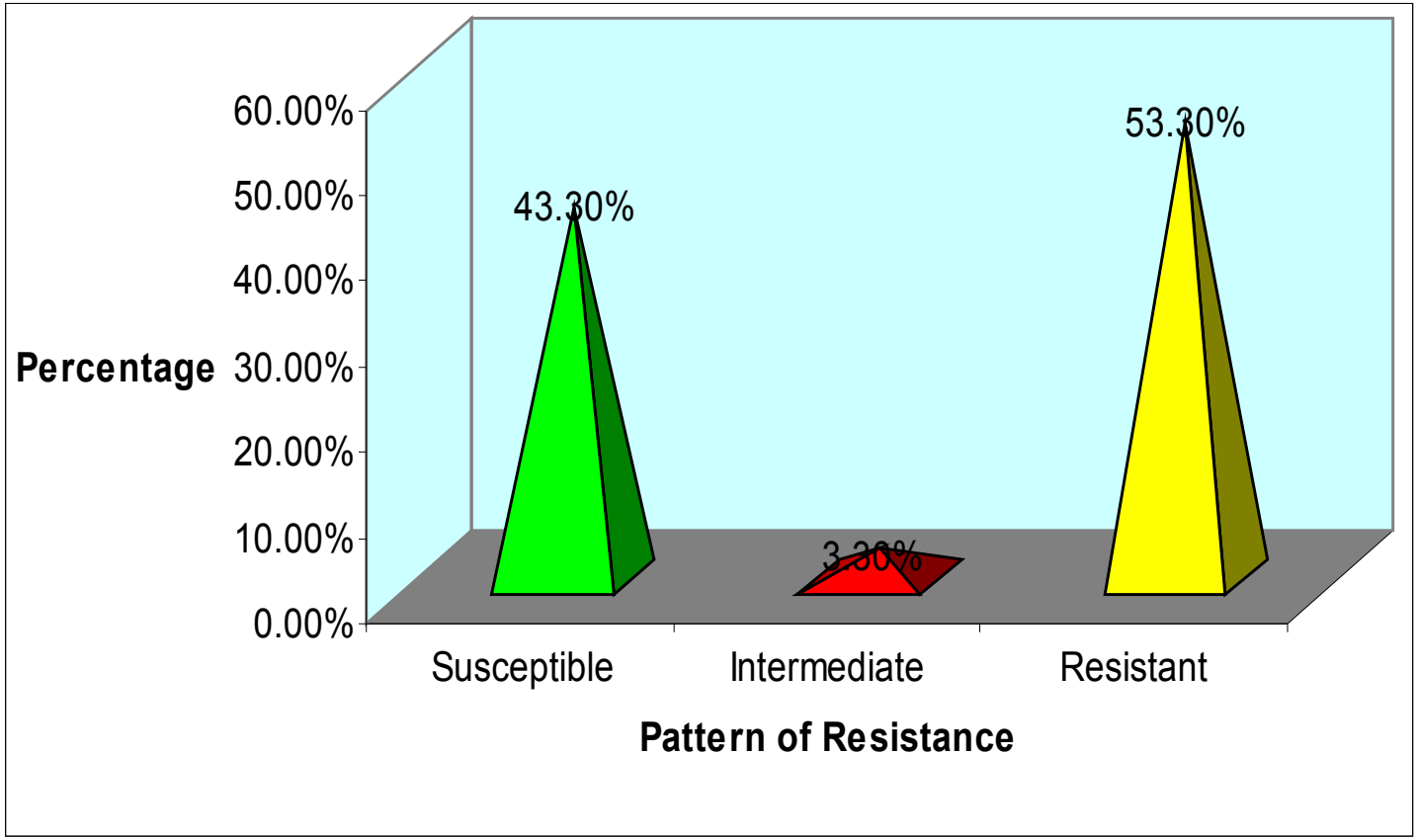


FIGURE – 5

RESULTS OF METHICILLIN RESISTANCE IN S.AUREUS AS DETERMINED BY CEFOXITIN (30ug) DISC DIFFUSION METHOD

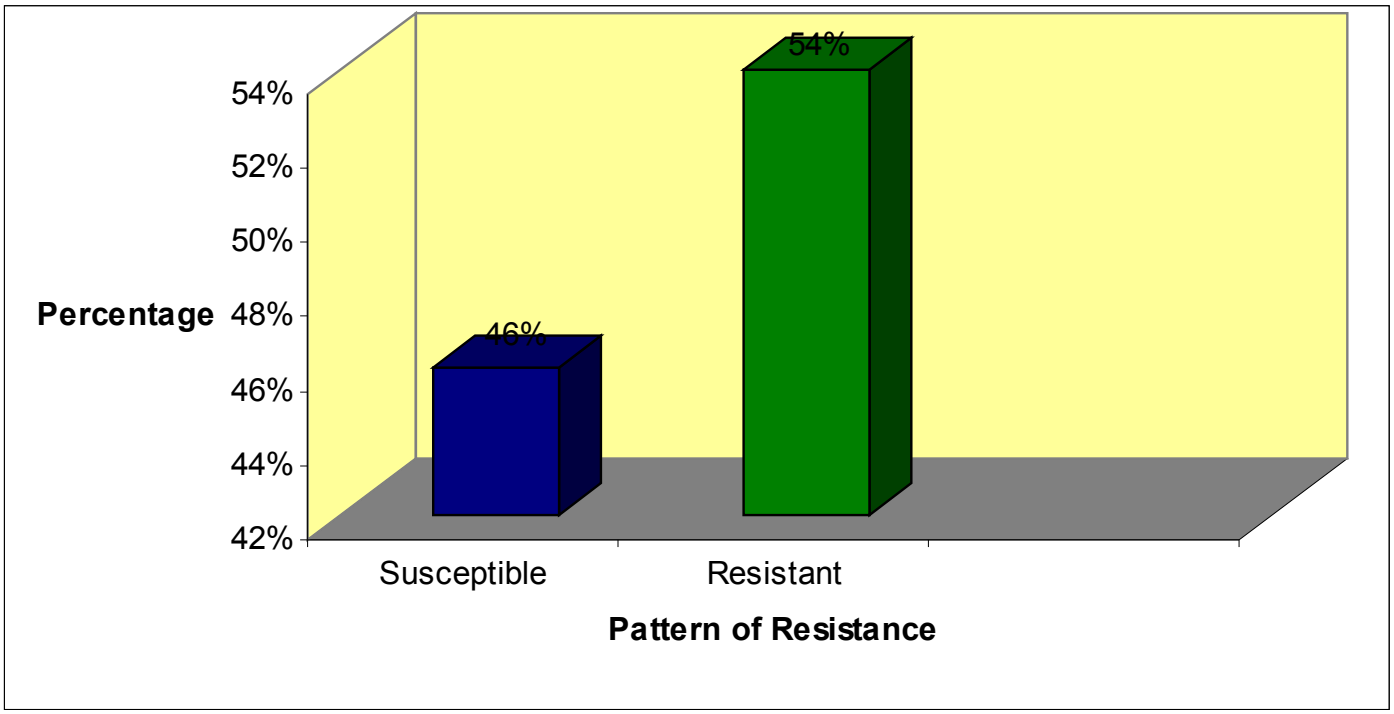


FIGURE – 6

RESULTS OF METHICILLIN RESISTANCE IN S.AUREUS AS DETERMINED BY MIC OF OXACILLIN – MICROBROTH DILUTION METHOD

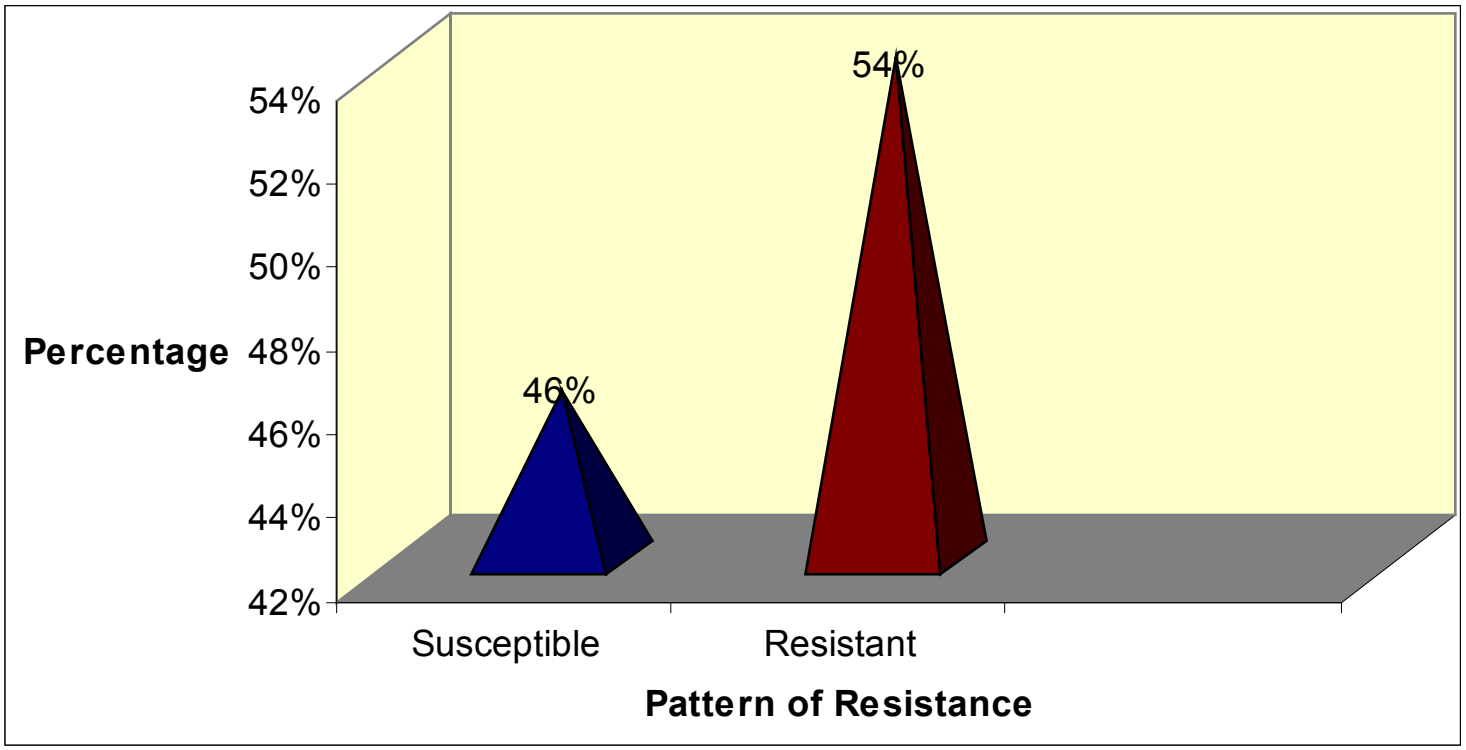
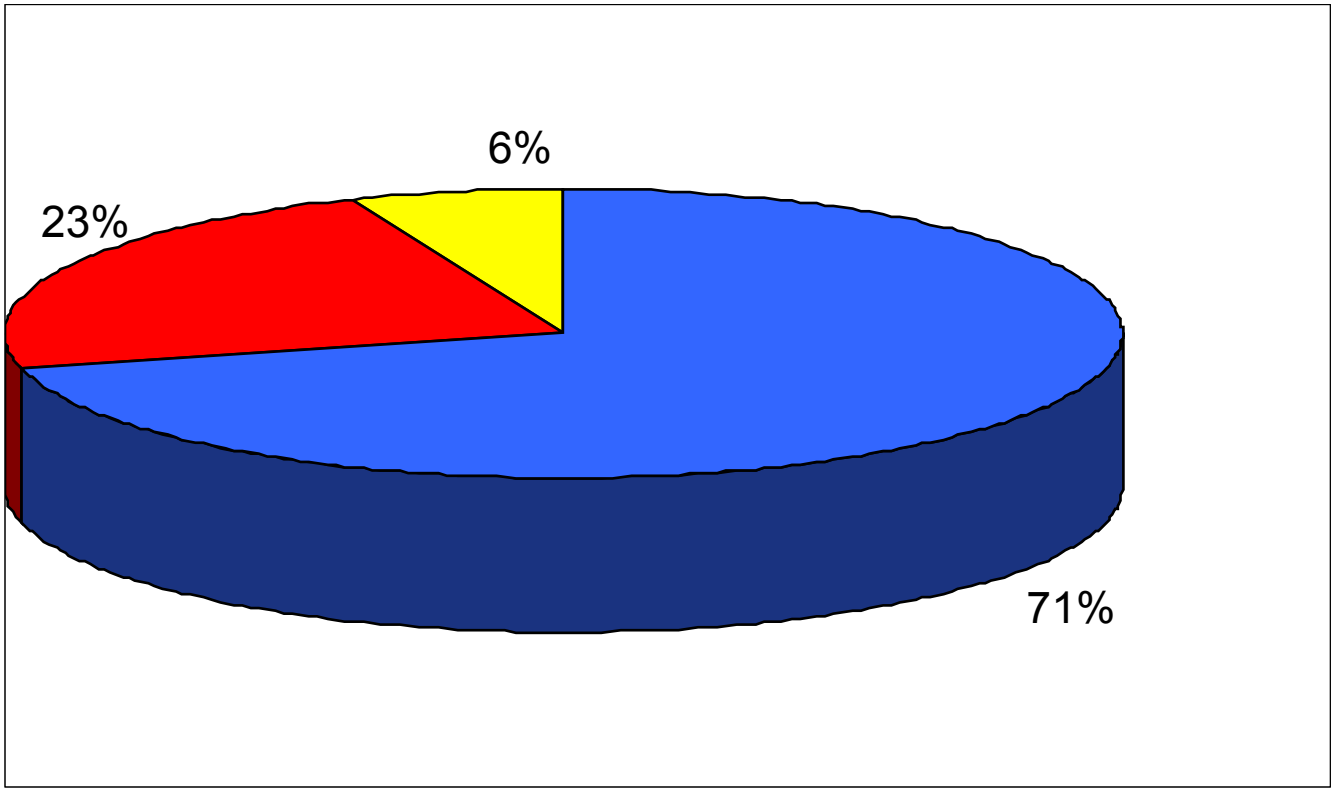


FIGURE – 7

DISTRIBUTION OF OXACILLIN MIC VALUES AMONG S.AUREUS



High Level : ≥ 32µg/ml
Moderate Level : ≤ 16 ≥ 8 µg/ml
Low Level 4µg /ml

FIGURE – 8

RESULTS OF METHICILLIN RESISTANCE IN S.AUREUS AS DETERMINED BY OXACILLIN SCREEN AGAR (6µg/ML – OXACILLIN)

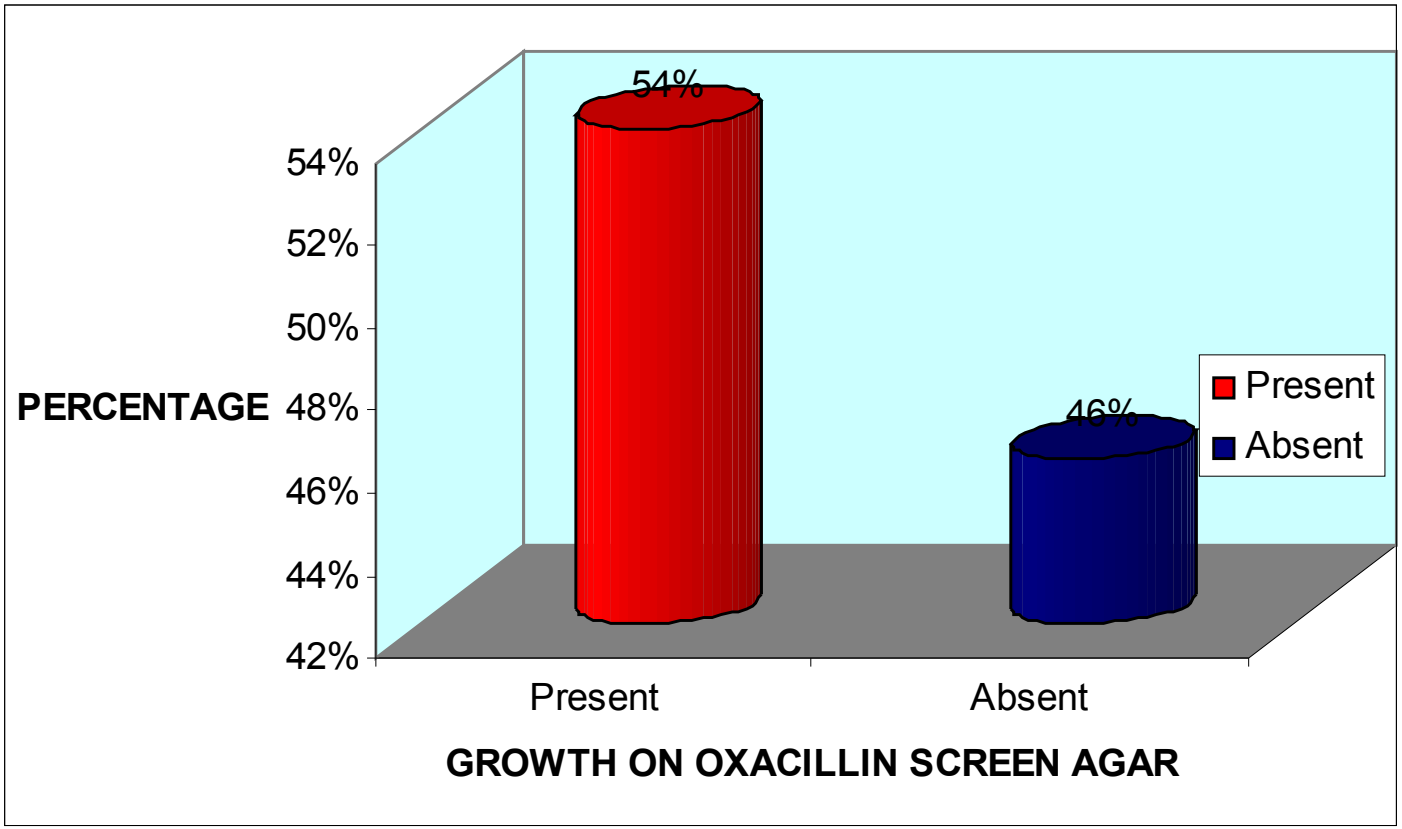


FIGURE – 9

RESULTS OF mecA GENE DETECTION BY PCR N=50

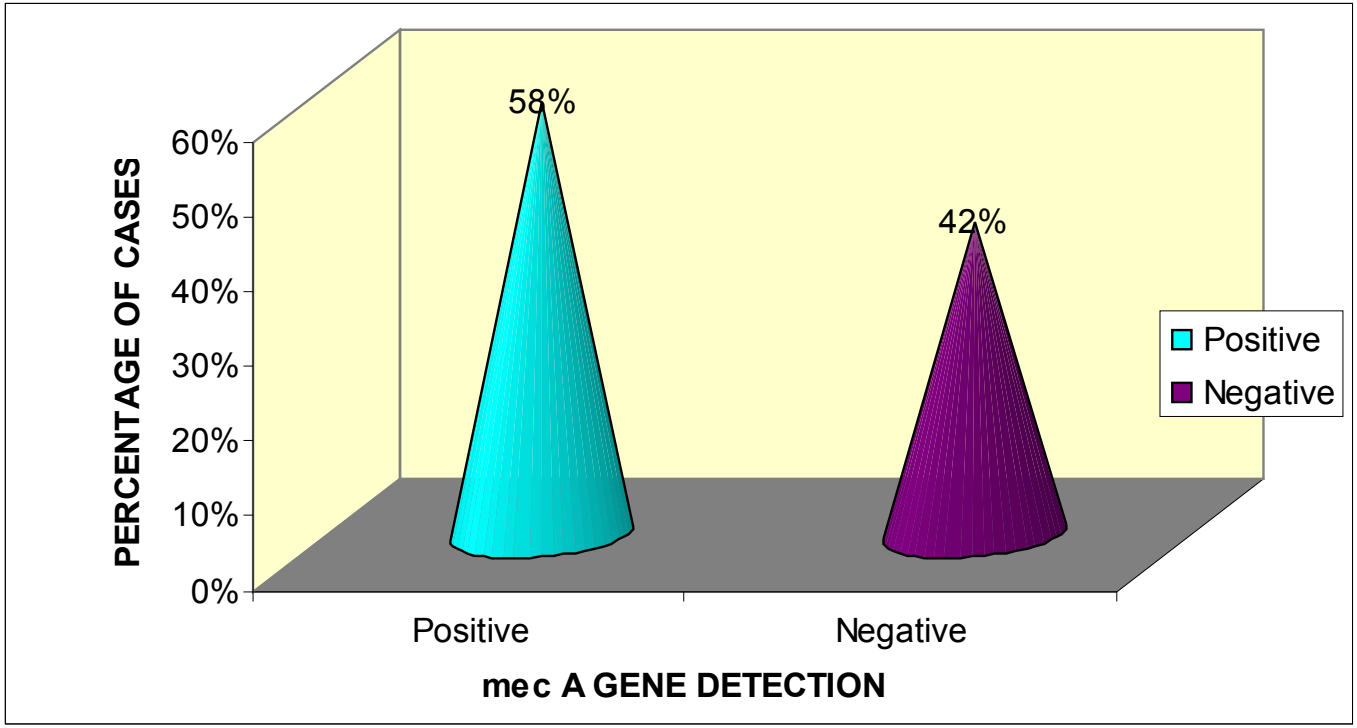


FIGURE – 10

ANTI MICROBIAL RESISTANT PATTERN OF MRSA N=81

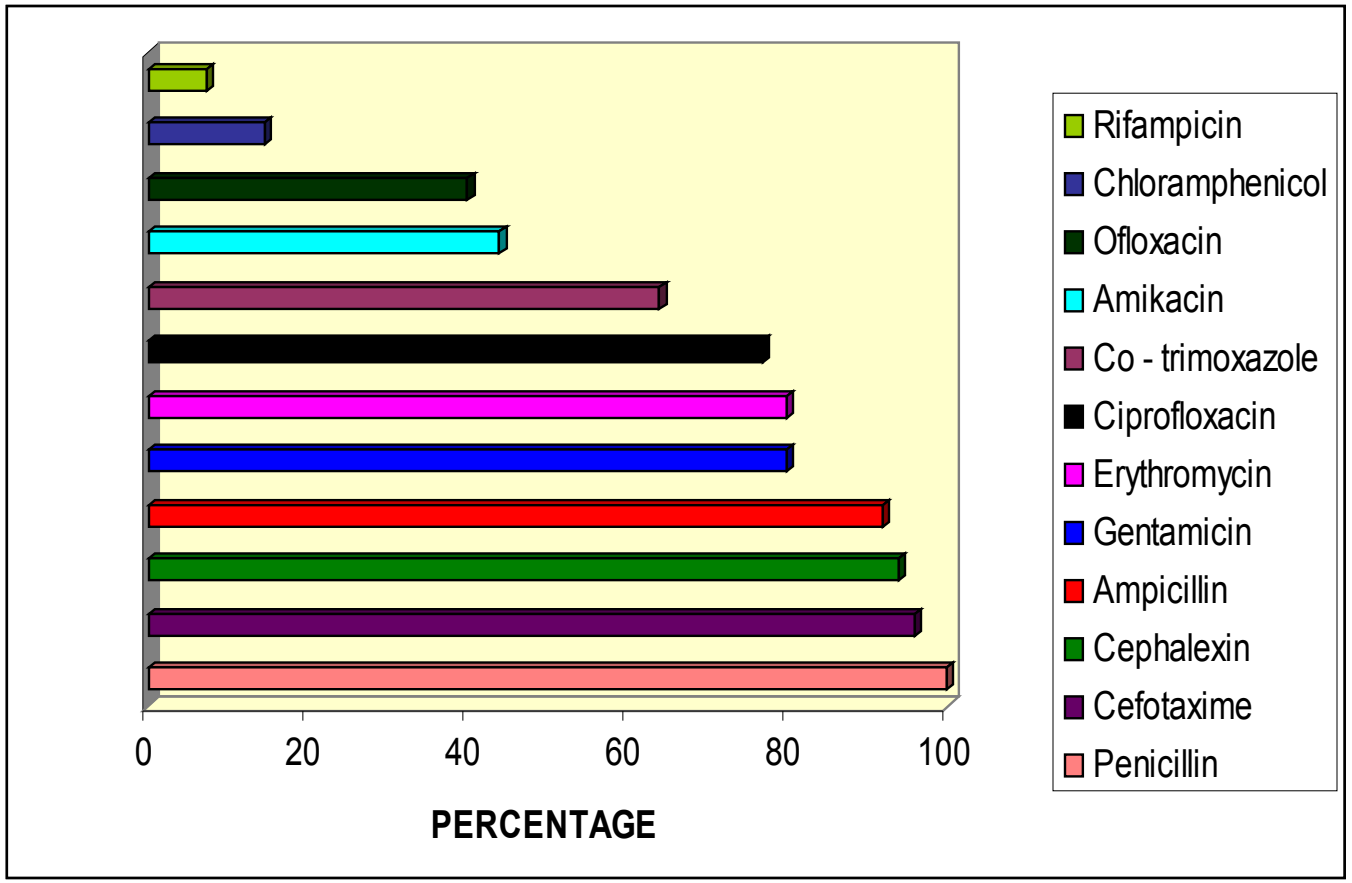
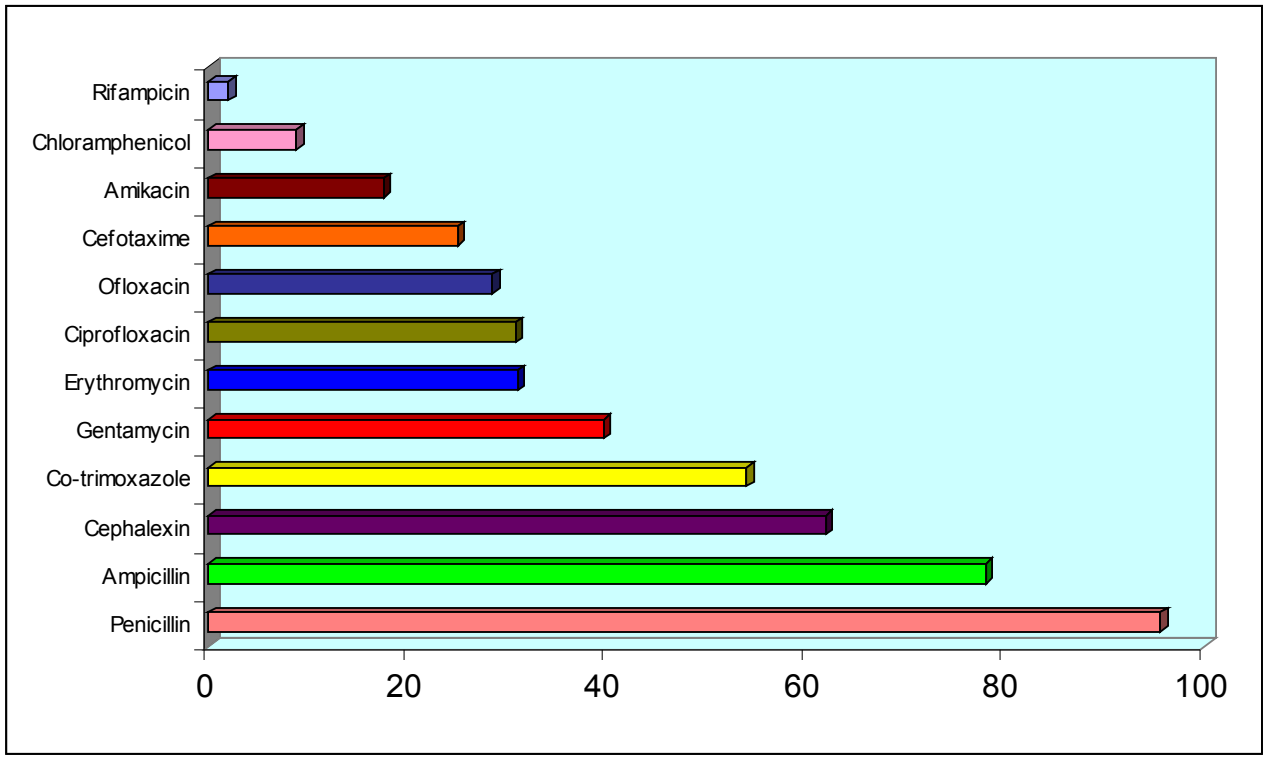


FIGURE – 11

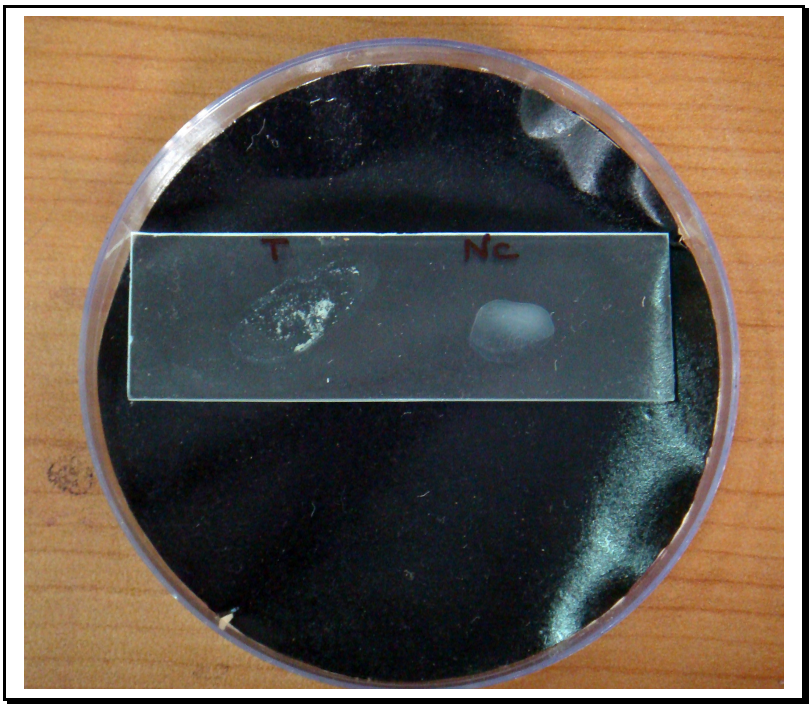
ANTI MICROBIAL RESISTANT PATTERN OF MSSA N=69


Rifampicin
Chloramphenicol
Amikacin
Cefotaxime
Ofloxacin
Ciprofloxacin
Erythromycin
Gentamycin
Co-trimoxazole
Cephalexin
Ampicillin
Penicillin
0
20
40
60
80
100

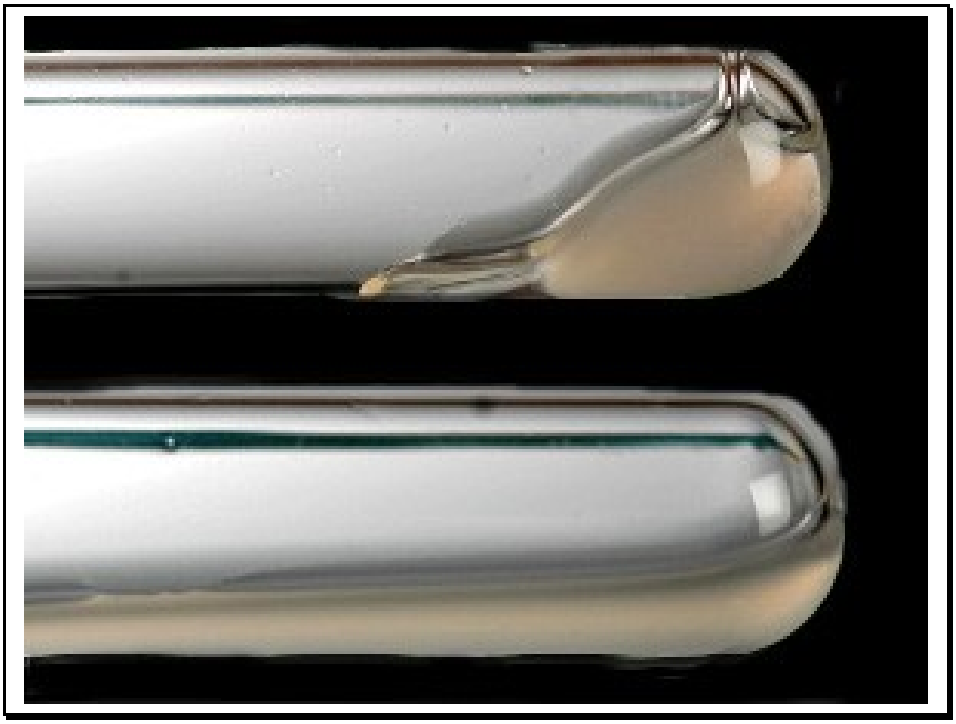
Colour Plates

NUTRIENT AGAR PLATE WITH WHITE OPAQUE COLONIES

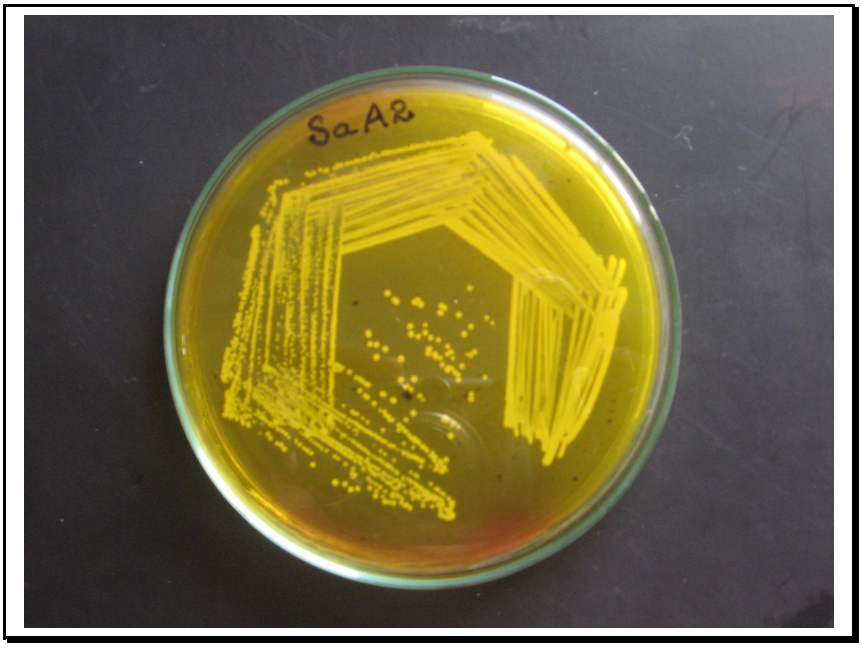
SLIDE COAGULASE - TEST



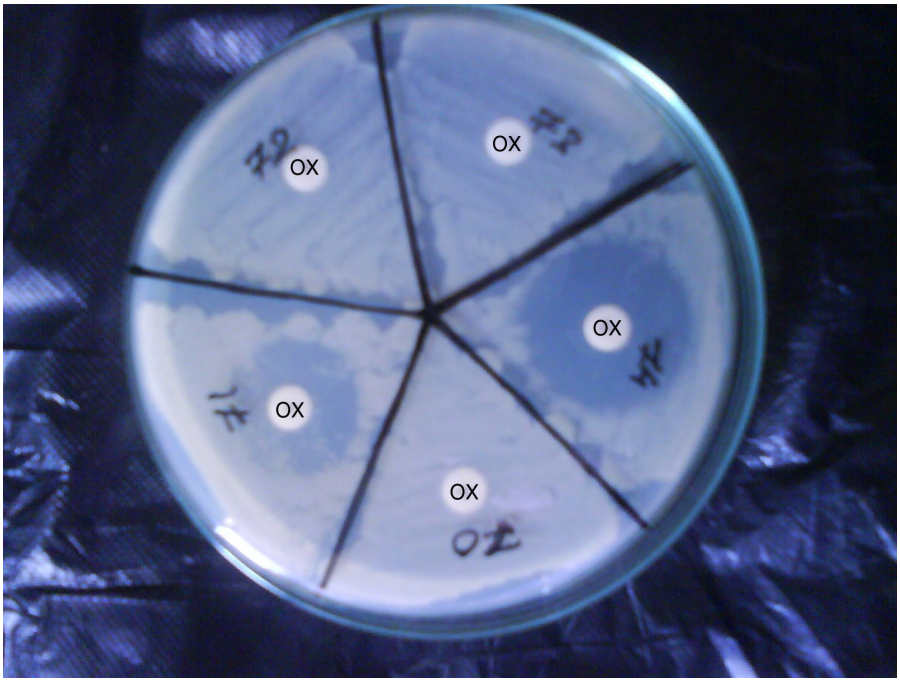
TUBE COAGULASE TEST



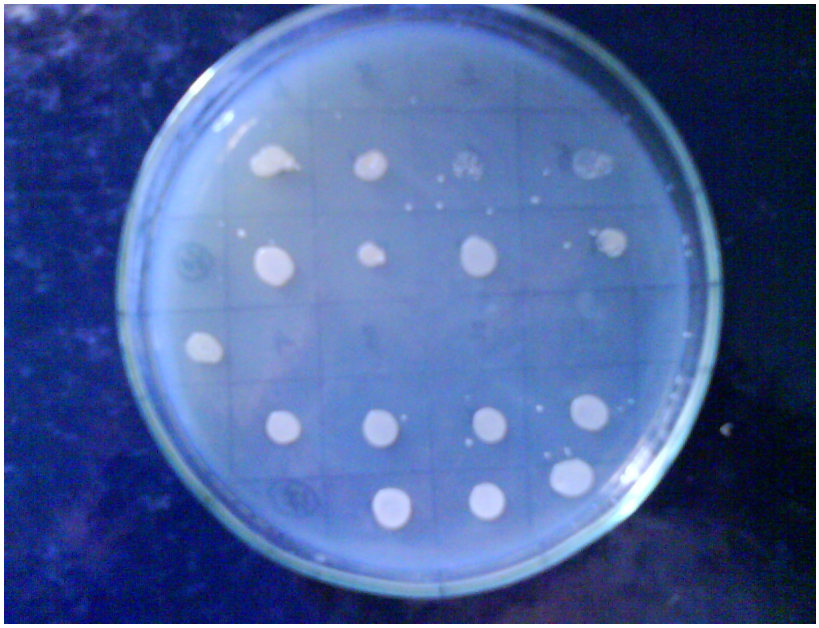
MANNITOL SALT AGAR WITH YELLOW COLONIES



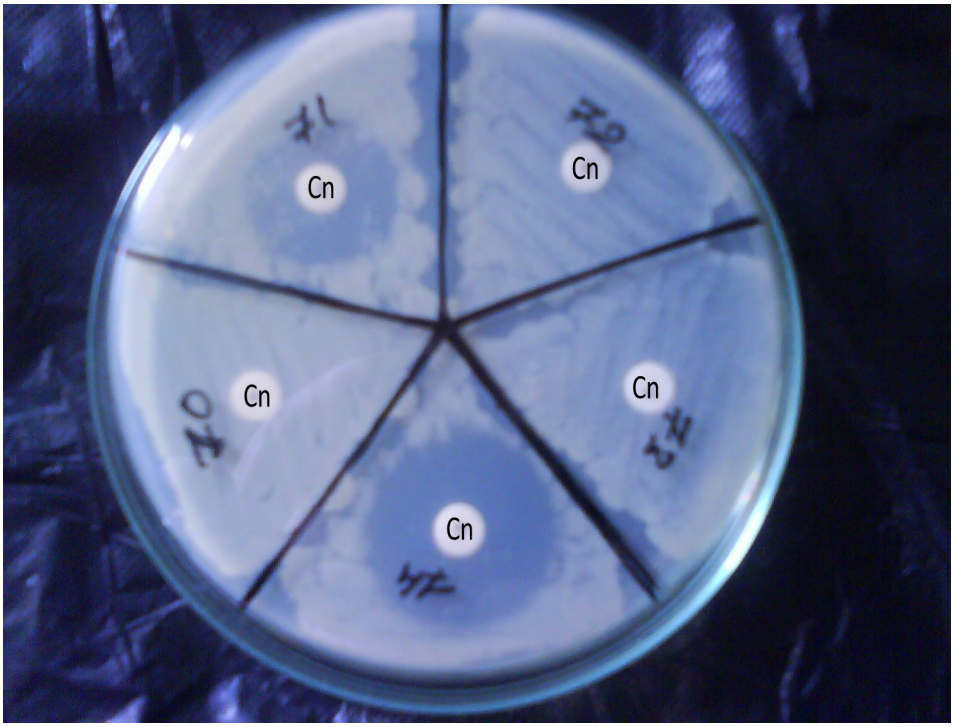
OXACILLIN DISC DIFFUSION METHOD



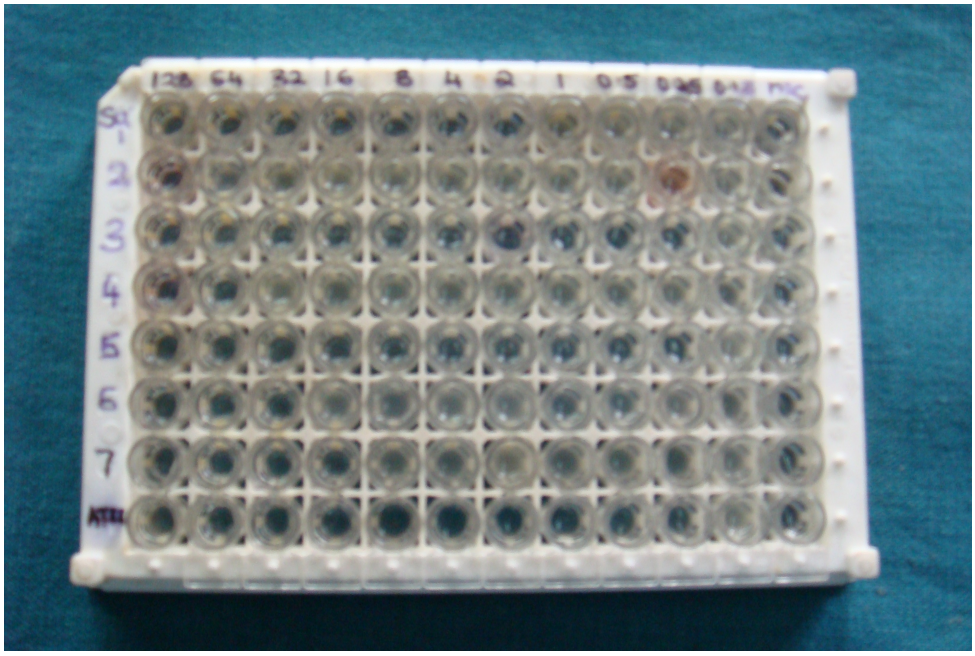
OXACILLIN SCREEN AGAR



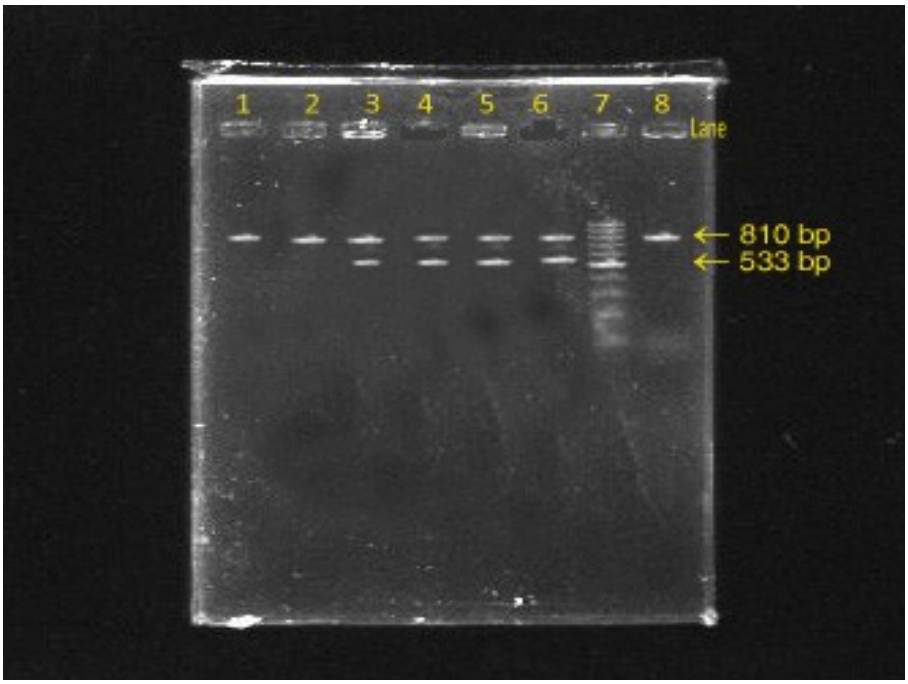
CEFOXITIN - DISC DIFFUSION METHOD



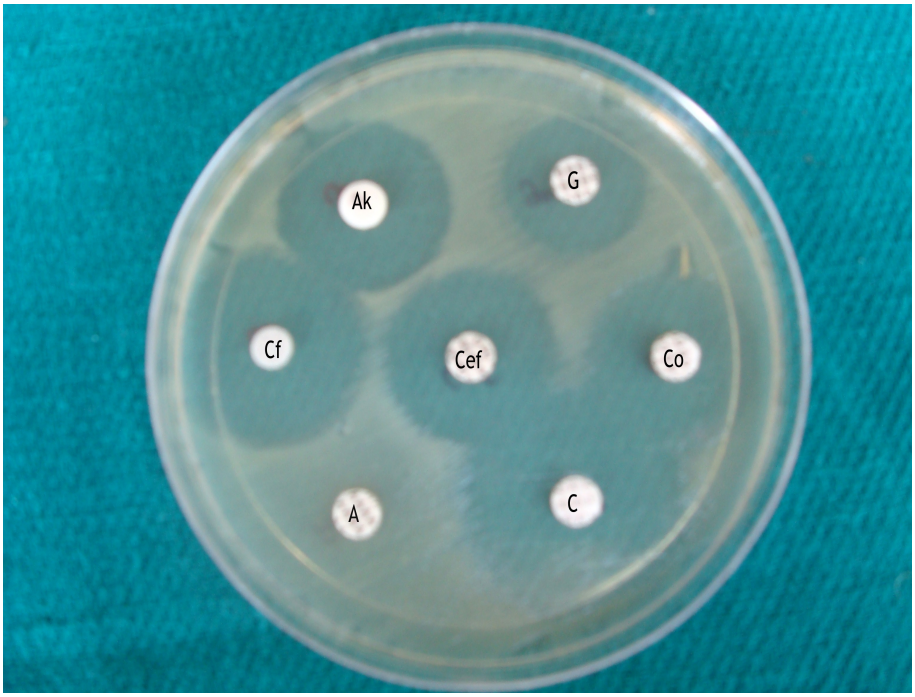
MIC - OXACILLIN - MICROBROTH DILUTION METHOD



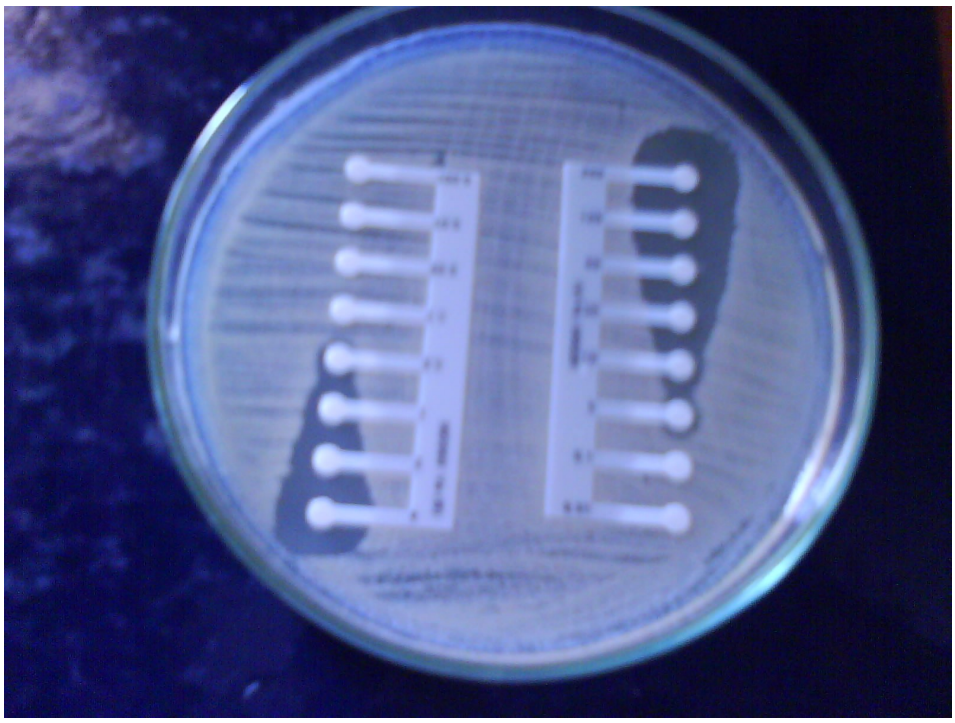
MULTIPLEX PCR - mec A & coag gene



ANTIBIOGRAM



E - TEST FOR VANCOMYCIN



Discussion

DISCUSSION

Methicillin Resistant *Staphylococcus aureus* (MRSA) is a major nosocomial pathogen causing significant morbidity and mortality. The epidemiology of MRSA has continued to evolve since its first appearance more than three decades ago. Epidemic strains of these MRSA are usually resistant to several other antibiotics. During the past 15 years, the appearance and world wide spread of many such clones have cuased major therapeutic problems in many hospitals as well as diversion of considerable resources to attempts at controlling their spread.

Hence this study was done to detect MRSA isolates from patients with soft tissue infection and to determine its antimicrobial susceptibility pattern.

A total of 150 *S. aureus* were isolated from soft tissue infection during the study period Jan 2007 to Jan 2008.

In the present study, 71.3% of isolates were from male and 28.7% were from females. (Table-1).In the study conducted by Christian et al in 2007, 60% isolates were from males and 40% were from females[22]

The maximum number of isolates in this study were in the age group of 21 – 40 (56%) followed by 28% in 41 – 60 years age group.

(Table-2).This was similar to the study conducted by Prakash et al in 2007, where 55% of the isolates were from 21 – 40 years age group[81].

In the present study, *S. aureus* isolates were predominantly from orthopedics department 51 (34%) followed by general surgery department 42 (28%) .(Table-3).This was similar to the study conducted Srinivasan et al in 2006, where the maximum number of isolates were from orthopedics department (28%) followed by General Surgery (26%)[97].

Among 150 isolates of *S,aureus,* 80 strains (53.3%) strains were methicillin resistant , 5 strains (3.3%) were intermediate and 65 strains (43.3%) were susceptible as determined by oxacillin disc diffusion method.(Table-4) denoting that oxacillin disc diffusion was not reliable for the identification of intermediate strains.In the study conducted by Erics et al in 2008, 42.7% strains were methicillin resistant , 51.6% strains were susceptible and 5.6% strains were intermediate as detected by oxacillin disc diffusion method[33].

In the present study, the results of methicillin resistance in *S. aureus* determined by cefoxitin disc (30 µg) diffusion method showed that 46% of strains were susceptible and 54% of strains were resistant. (Table-5) Rahbar et al in 2006 detected 32% of strains as methicillin resistant and 68% of strains as susceptible by cefoxitin disc diffusion

method[85]. Cefoxitin is a better inducer of *mec A* gene and disc diffusion tests using cefoxitin give clearer end points and are easier to read than tests with oxacillin.

The present study reveals that using cefoxitin is a good alternative method for oxacillin disc diffusion method for detection of MRSA especially in the identification of intermediate resistant strains of *S. aureus*.

The minimum inhibitory concentration of oxacillin as determined by microbroth dilution method in the present study demonstrated 81 (54%) strains as resistant and 69 (46%) as susceptible.(Table-6). In the study conducted by A. Chaudhury et al in 2008, 52.8% of *S. aureus* (MIC ≥ 4 µg/ml) were resistant by microbroth dilution technique[21].

High level oxacillin resistance ≥32 µg/ml were seen in 58 strains (38.7%). Moderate level oxacillin resistance ≤ 16 ≥ 8 were seen in 19 strains (12.6%). Low level oxacillin resistance of 4 µg/ml were seen in 5 strains (3%).(Table-8).

In the present study, 69 strains (46%) and 81 strains (54%) of *S. aureus* were determined to be susceptible and resistant strains by the oxacillin screen agar method respectively.(Table-9).In the study

conducted by G K Bhat et al in 2007, 33% strains of *S aureus* were methicillin resistant by oxacillin screen agar method[11].

The conventional MRSA detection assays are simple and relatively cheap in detecting methicillin resistance. Accurate determination of MRSA by conventional methods are subjected to variation in inoculum size, incubation time, temperature, pH and salt concentration. It is in such instance that detection of *mec A* gene is useful by molecular methods[85].

In the present study, due to high cost, PCR was restricted to 50 isolates. Multiplex PCR was used to detect the presence of *mecA* gene and *coag* gene in 50 isolates.In this study, *mec A* gene was detected in 29 isolates (58%) (table – 11). The *coag* gene was present in all isolates. (Table 10).In the study conducted by Raalpalli et al 2008, 45.3% of strains were detected as MRSA by multiplex PCR using mecA[84].

In this study on comparing the phenotypic and genotypic results of the 50 isolates (Table – 12) the phenotypic methods such as oxacillin screen agar, cefoxitin disc diffusion, MIC- Microbroth dilution method had sensitivityof 90% specificity of 100% and accuracy of 94%. The phenotypic results are therefore comparable with that of the *mec A* – the gold standard.

In the study conducted by George et al 2001, the oxacillin screen agar had a sensitivity of 99% and specificity of 98.1% and the microbroth dilution had a sensitivity of 99% and specificity of 100% when compared with *mec A* the gold standard[39].In the study conducted by Jana et al 2005, the cefoxitin disc diffusion method had 100% sensitivity and specificity when compared with that of *mec A* gene[51].

In the present study, the antimicrobial susceptibility pattern of MRSA and MSSA isolates were determined by disc diffusion method. Among the MRSA isolates, 100% strains were resistant to penicillin, 92% to ampicillin, 94% to cephalexin, 95.8% to cefotaxime, 80% to erythromycin, 80% to gentamicin, 77% to ciprofloxacin and 84% to rifampicin. Moderate level of resistance was seen to co-trimoxazole (64%), Amikacin (44%) and ofloxacin (40%). However all MRSA strains were sensitive to vancomycin, teicoplanin and linezolid.

In the study conducted by Anupurba et al 2003, more than 80% of MRSA were found to be resistant to majority of antibiotics tested like cephalexin, ciprofloxacin, penicillin, co – trimoxazole, gentamicin, erythromycin and tetracycline and 60.5% to amikacin. No strains were resistant to vancomycin.

Among the MSSA isolates, 95.6% strains were resistant to penicillin, 88% to ampicillin, 62% to cephalexin, 25% to cefotaxime, 31% to erythromycin, 39.7% to gentamicin 17.6% to Amikacin, 30.8% to ciprofloxacin and 48.5% to ofloxacin. All these isolates were sensitive to vancomycin, teicoplanin and linezolid. In the study conducted by S. Vidhani et al 2001,among the MSSA isolates 94.5%were resistant to penicillin,89%to amoxycillin' 32%to amikacin and 22%to erthyromycin and cefotaxime,All the MSSA isolates were sensitive to vancomycin[108].

In the present study ,the antibiotic sensitivity results show that all MRSA isolates were more resistant to antibiotics as compared to MSSA isolates which was similar to the study conducted by Vidhani et al 2001[108].

In the present study, all strains (150) of *S. aureus* were sensitive to vancomycin by the disk diffusion method which was similar to the study conducted by Rajadurai Pandi et al in 2006 [83].E-test detected all isolates of *S aureus* in the present study as vancomycin sensitive. In the study conducted by Hakim et al 2007,13%of the isolates were detected as VISA by E –test method. .

Summary & Conclusion

SUMMARY

1) Among 150 *S. aureus* isolated form pus samples, maximum number of cases were in the age group (21 – 40 years)

2) There was a male preponderance among the isolates. (71.3%)

3) Majority of samples were from orthopedics department (34%) followed by general surgery (28%)

4) Oxacillin disc diffusion method detected 53.3% strains as methicillin resistant, 3.3% as intermediate and 43.3% strains as susceptible

5) 54% of the isolates were methicillin resistant and 46% were susceptible by cefoxitin disc diffusion method.

6) Minimum inhibitory concentration of oxacillin by microbroth dilution method showed that 54% of isolates as methicillin resistant and 46% of isolates as methicillin susceptible.

7) High level of oxacillin resistance were seen in 38.7%, moderate level in 12.6% and low level of oxacillin resistance in 3% of isolates.

8) Oxacillin screen agar detected 54% strains as methicillin resistant and 46% strains as susceptible.

9) Multiplex PCR detected 58% strains as methicillin resistant and 42% strains as susceptible

10) All the isolates (100%) were sensitive to vancomycin by disc diffusion method and E – test.

11) Antibiotic sensitivity results showed that MRSA strains were more resistant to antibiotics compared to methicillin sensitive *Staphylococcus aureus* (MSSA) isolates.

CONCLUSION

Among the *Staphylococcus aureus* isolated from patients with soft tissue infections, 54% were methicillin resistant. The conventional phenotypic methods in the detection of MRSA such as cefoxitin disc diffusion method, MIC – micro broth dilution method and oxacillin screen agar had a high degree of sensitivity and specificity when compared with that of mec A - the gold standard and hence it can be used in the detection of MRSA isolates.MRSA isolates were more resistant to many of the antimicrobials when compared with MSSA isolates. All the isolates were sensitive to vancomycin

Proforma

PROFORMA

Name	:	IP /OP No
Age	:	Ward
Sex	:	D.O.A.
Address	:	
Specimen	:	
Diagnosis of the patient	:	

Laboratory analysis:

Nutrient agar	:	
Blood agar	:	
Antibiogram		
PCR		

Signature of Microbiologist

Appendix

APPENDIX

Nutrient agar	-	Gms / litre
Peptic digest of animal tissue	-	5.00
Beef extract	-	1.50

Yeast extract	-	1.50
Sodium chloride	-	5.00
Agar	-	15.00

The above ingredients were suspended in 1 litre of water. Boild to dissolve the medium completely. Final pH adjusted to 7.4 ± 0.2. Sterilized by autoclaving at 121°C for 15 minutes.

Blood agar

Sterile defibrinated sheep blood	-	10 ml
Nutrient agar (melted)	-	100 ml

Sterile nutrient agar was melted, cooled to about 45 – 50°C, 7-10 ml of sterile defibrinated sheep blood was added, mixed well and 15 ml of blood agar was poured in petri dishes.

MacConkey Agar

Peptone	-	2gm
Nacl	-	2 gm
Bile salt	-	0.5 gm
Lactose	-	1 gm
Agar	-	1.5 gm
Distilled water	-	100 ml

All the ingredients except lactose were dissolved in distilled water by heating. pH adjusted to 7.6 1 ml to neutral red solution was added to every 100 ml of medium with lactose and sterilized by autoclaving at 121°C for 15 minutes.

Mueller – Hinton Agar

Beef infusion	-	300 ml
Caesin hydrolysate	-	17.5 g
Starch	-	1.5 g
Agar	-	10 g
Distilled water	-	1 litre

The ingredients were dissolved in one litre of distilled water, mixed thoroughly. Heated with frequent agitation and boiled for 1 minute. pH adjusted to 7.4 ; Sterilised by autoclaving and poured in plates.

Mannitol salt agar

Beef extract	-	0.1g
Peptone	-	1 g
Nacl	-	7.5 g
Mannitol	-	1g

Agar	-	1.5 g
Phenol red	-	0.0025
Distilled water	-	100 ml

The above ingredients were dissolved in 100ml of distilled water, mixed thoroughly. Heated with frequent agitation and pH adjusted to 7.6. Sterilized by autoclaving and poured in plates.

Oxacillin screen agar

1) Mueller Hinton agar - 100gms

2) Nacl – 4%

3) Distilled water – 1000 ml

4) Oxacillin – 6 µg / ml

Mueller Hinton agar was dissolved in 1 litre of distilled water and Nacl – 4 gms/100ml was added to it. Mixid thoroughly. Sterlised by autoclaving. Cooled to about 45 – 50°C – Oxacillin solution 6 µg/ml is added to it and poured in plates.

DNase agar

1) Tryptose - 20g

2) Deoxyribonucleic acid – 2 g

3) Sodium chloride – 5 g

4) Agar Powder – 12 g

All the ingredients were dissolved in one litre of distilled water and mixed thoroughly. Sterilized by autoclaving and poured in plates.

Abbreviations

ABBREVIATIONS

MRSA	-	Methicillin resistant *Staphylococcus aureus*
TSST	-	Toxic shock Syndrome toxin
Agr	-	Accessory global regulator
Sar	-	Staphylococcal accessory regulator

PBP	-	Penicillin Binding protein
SCC	-	Staphylococcal Cassette Chromosome
fem	-	factors essential for methicillin resistance
VISA	-	Vancomycin Intermediate *Staphylococcus aureus.*
VRSA	-	Vancomycin Resistant *Staphylococcus aureus.*
PCR	-	Polymerase Chain Reaction
bp	-	base pair

INSTITUTIONAL ETHICAL COMMITTEE
GOVERNMENT GENERAL HOSPITAL & MADRAS MEDICAL COLLEGE,
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K.Dis.No.16328 P & D3/Ethies/Dean/GGH/08 Dated: **08.09.2008**

Title of the work	: DETECTION OF METHICILLIN RESISTANT STRAINS OF STAPHYLOCOCCUS AUREUS USING PHENOTYPIC AND GENOTYPIC METHODS IN A TERTIARY CARE HOSPITAL.
Principal Investigator	: **Dr.N. Lakshmi Priya**
Department	: **Institute of Microbiology, MMC, Chennai - 3**

The request for an approval from the Institutional Ethical Committee (IEC) was considered on the IEC meeting held on 10th Sept. 2008 at 2 p.m in GGH, Dean's Chamber, Chennai – 3.

The members of the Committee, the Secretary and the Chairman are pleased to approve the proposed work mentioned above, submitted by the principal investigator.

The principal investigator and their term are directed to adhere the guidelines given below:

1. You should get detailed informed consent from the patients/participants and maintain confidentiality.
2. You should carry out the work without detrimental to regular activities as well as without extra expenditure to the Institution or Government.
3. You should inform the IEC in case of any change of study procedure, site and investigation or guide.
4. You should not deviate form the area of the work for which I applied for ethical clearance.
5. You should inform the IEC immediately in case of any adverse events or serious adverse reactions.
6. You should abide to the rules and regulations of the institution(s)
7. You should complete the work within the specific period and if any extension of time is required, you should apply for permission again and do the work.
8. You should submit the summary of the work to the ethical committee on completion of the work.
9. You should not claim funds from the Institution while doing the work or on completion.
10. You should understand that the members of IEC have the right to monitor the work with prior intimation.

SECRETARY
IEC, GGH,CHENNAI

CHAIRMAN
IEC, GGH,CHENNAI

DEAN
GGH &MMC, CHENNAI

RKM.5.6(2)

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